TRANSFORMED CELL WITH ENHANCED SENSITIVITY TO ANTIFUNGAL COMPOUND AND USE THEREOF

BACKGROUND OF THE INVENTION

Field of the invention

The present invention relates to a transformed cell with enhanced sensitivity to an antifungal compound and use thereof.

Description of the related art

It is known that, when a fungicide containing a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" or a phenylpyrrole antifungal compound as an active ingredient is acted on a certain plant-pathogenic filamentous fungus, glycerol synthesis in a cell is stimulated in the fungus like as when undergoes high osmotic stress, and the fungus can not control an intracellular osmolarity, leading to death. From such the activity to the plant-pathogenic filamentous fungus, a protein in a signal transduction system which is involved in osmolarity response was predicted as a target protein of an antifungal compound contained in these fungicides as an active ingredient.

In Neurospora crassa exhibiting sensitivity to the aforementioned antifungal compound, an osmosensitive mutant os-1 was reported. This mutant os-1 exhibited resistance to the aforementioned antifungal compound and, by analysis of the

mutant, an os-1 gene which is an osmosensing histidine kinase gene was isolated as a causative gene. A protein having an amino acid sequence encoded by a nucleotide sequence of this os-1 gene was a protein which has a structure of histidine kinase of a two-component regulatory system and, at the same time, has a characteristic region (hereinafter, referred to repeat sequence region in some cases) in which amino acid sequences composed of about 90 amino acids and having homology to each other are present repetitively about 6 times (see, for example, U.S.P. NO 5,939,306; Genebank accession U50263, U53189, AAB03698, AAB01979; Alex, A.L. et al., Proc. Natl. Acd. Sci. USA 93:3416-3421; Schumacher, M.M. et al., Current Microbiology 34:340-347; Oshima, M. et al., Phytopathology 92 (1):75-80; Fijimura, M. et al., J. Pesticide Sci. 25:31-36). A gene having homology to the os-1 gene was also isolated from plant-pathogenic filamentous fungus such as Botryotinia fuckeliana, Magnaporthe grisea, Fusarium solani and the like, and its nucleotide sequence and an amino acid sequence encoded by the gene are published. It is known that genes having homology with the os-1 gene are specifically present in filamentous fungus among eukaryotic organisms (see, for example, GeneBank accession AF396827, AF435964, AAL37947, AAL30826; Fujimura, M. et al., Pesticide Biochem. Physiol. 67:125-133; GeneBank accession AB041647, BAB40497).

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method of detecting the antifungal activity and a method of selecting an antifungal compound using the os-1 gene and a gene having homology with the gene.

Under such the circumstances, the present inventor intensively studied and, as a result, found a transformed cell with enhanced sensitivity to an antifungal compound, and found a method of detecting the antifungal activity using this transformed cell and a method of selecting an antifungal compound using this transformed cell, which resulted in completion of the present invention.

Thus, the present invention provides:

1. A transformed cell in which a polynucleotide having a nucleotide sequence encoding an amino acid sequence of an osmosensing histidine kinase having no transmembrane region is introduced in a functional form into a cell deficient in at least one hybrid-sensor kinase;

2. The transformed cell according to the above 1, the polynucleotide having a nucleotide sequence encoding an amino acid sequence of an osmosensing histidine kinase having no transmembrane region is a polynucleotide complementing the deficiency in hybrid-sensor kinase in the cell deficient in at least one hybrid-sensor kinase in which the polynucleotide is introduced;

3. The transformed cell according to the above 1, wherein the cell is a microorganism;

4. The transformed cell according to the above 3, wherein the microorganism is budding yeast;

5. The transformed cell according to the above 1, wherein the osmosensing histidine kinase having no transmembrane region is an osmosensing histidine kinase having no transmembrane region and having a mutation which confers resistance to any of a dicarboxyimide antifungal compound , an "aromatic hydrocarbon antifungal compound" and a phenylpyrrole antifungal compound to the cell;

6. The transformed cell according to the above 5, wherein the osmosensing histidine kinase having no transmembrane region is a histidine kinase having the amino acid sequence represented by SEQ ID NO: 13;

7. The transformed cell according to the above 1, wherein the osmosensing histidine kinase having no transmembrane region is an osmosensing histidine kinase which is derived from a plant-pathogenic filamentous fungus and has no transmembrane region;

8. The transformed cell according to the above 1, wherein the osmosensing histidine kinase having no transmembrane region is an osmosensing histidine kinase which is derived from Botryotinia fuckeliana, Magnaporthe grisea, Fusarium oxysporum, Mycospharella tritici, Thanatephorus cucumeris or Phytophthora

infestans, and has no transmembrane region;

9. The transformed cell according to the above1, wherein the osmosensing histidine kinase having no transmembrane region is an osmosensing histidine kinase having no transmembrane region which has an amino acid sequence represented by SEQ ID NO: 1, SEQ ID NO: 16, SEQ ID NO: 41, SEQ ID NO: 55, SEQ ID NO: 68 or SEQ ID NO: 90;

10. The transformed cell according to the above 1, wherein the nucleotide sequence encoding an amino acid sequence of the osmosensing histidine kinase having no transmembrane region is a nucleotide sequence represented by SEQ ID NO: 2, SEQ ID NO: 17, SEQ ID NO: 42, SEQ ID NO: 56 or SEQ ID NO: 69;

11. A method of assaying the antifungal activity of a substance, which comprises:

a first step of culturing the transformed cell as defined in the above 1 in the presence of a test substance;

a second step of measuring an amount of intracellular signal transduction from the osmosensing histidine kinase having no transmembrane region expressed in the transformed cell cultured in the first step or an index value having the correlation therewith; and

a third step of assessing the antifungal activity of the test substance based on a difference between an amount of intracellular signal transduction or an index value having the correlation therewith measured in the second step and a control;

12. The method of assaying according to the above 11, wherein the amount of intracellular signal transduction from the osmosensing histidine kinase having no transmembrane region or the index value having the correlation therewith is an amount of growth of the transformed cell;

13. A method of searching an antifungal compound, which comprises selecting an antifungal compound based on the antifungal activity assessed in the assaying method as defined in the above 11;

14. An antifungal compound selected by the searching method as defined in the above 13;

15. An osmosensing histidine kinase having no transmembrane region, wherein the kinase is derived from a plant-pathogenic filamentous fungus;

16. An osmosensing histidine kinase having no transmembrane region, which has an amino acid sequence selected from the group consisting of:

(a) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which has an amino acid sequence homology of 95% or more to the amino acid sequence represented by any of SEQ ID NOs: 41, 55 and 68;

(b) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Fusarium oxysporum-derived cDNA as a template and using an oligonucleotide

having the nucleotide sequence represented by SEQ ID NO: 52 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 53 as primers;

(c) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Mycospharella tritici-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 64 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 65 as primers;

(d) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Thanapethorus cucumeris-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 85 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 86 as primers;

(e) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is derived from Phytophthora infestans and has the amino acid sequence represented by SEQ ID NO: 90;

(f) the amino acid sequence represented by SEQ ID NO: 41;

(g) the amino acid sequence represented by SEQ ID NO: 55; and

(h) the amino acid sequence represented by SEQ ID NO: 68.

17. An osmosensing histidine kinase having no transmembrane

region, which has an amino acid sequence represented by SEQ ID NO: 41, SEQ ID NO: 55 or SEQ ID NO: 68;

18. A polynucleotide having a nucleotide sequence encoding an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, wherein the kinase is derived from a plant-pathogenic filamentous fungus;

19. A polynucleotide having a nucleotide sequence encoding an amino acid sequence selected from the group consisting of:

(a) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which has an amino acid sequence homology of 95% or more to the amino acid sequence represented by any of SEQ ID NOs: 41, 55 and 68;

(b) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Fusarium oxysporum-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 52 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 53 as primers;

(c) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Mycospharella tritici-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 64 and an oligonucleotide having the nucleotide sequence represented

by SEQ ID NO: 65 as primers;

(d) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Thanapethorus cucumeris-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 85 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 86 as primers;

(e) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is derived from Phytophthora infestans and has the amino acid sequence represented by SEQ ID NO: 90;

(f) the amino acid sequence represented by SEQ ID NO: 41;

(g) the amino acid sequence represented by SEQ ID NO: 55; and

(h) the amino acid sequence represented by SEQ ID NO: 68;

20. A polynucleotide having a nucleotide sequence represented by SEQ ID NO: 42, SEQ ID NO: 56 or SEQ ID NO:69;

21. A method of obtaining a polynucleotide having a nucleotide sequence encoding an amino acid sequence of osmosensing histidine kinase which is derived from a plant-pathogenic filamentous fungus and has no transmembrane region, which comprises a step of amplifying a desired polynucleotide by Polymerase Chain Reaction using an oligonucleotide having a nucleotide sequence represented by any of SEQ ID NOs: 30 to 40, 52, 53, 64, 65, 85 and 86 as primers, and a step of recovering the amplified desired

polynucleotide; and

22. An oligonucleotide which comprises a nucleotide sequence represented by any of SEQ ID NOs: 30 to 40, 52, 53, 64, 65, 85 and 86.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

The present invention will be explained in detail below.

The "transformed cell in which a polynucleotide having a nucleotide sequence encoding an amino acid sequence of an osmosensing histidine kinase having no transmembrane region is introduced in a functional form into a cell deficient in at least one hybrid-sensor kinase" is obtained by introducing a polynucleotide having a nucleotide sequence encoding an amino acid sequence of an "osmosensing histidine kinase having no transmembrane region" in a functional form into a "cell deficient in at least one hybrid-sensor kinase" which is a host cell. Herein, "introduction of a polynucleotide in a functional form" means that the polynucleotide is introduced so as to complement the deficiency in hybrid-sensor kinase, in other words, that the polynucleotide is introduced in such a form that a phenotype of the cell caused by the deficiency in hybrid-sensor kinase revert to a phenotype without the deficiency in hybrid-sensor kinase. Specifically, for example, in the case of budding yeast (e.g. Saccharomyces cerevisiae), when SLN1 which is hybrid-sensor kinase is deleted, the SLN1-deficient yeast cell

shows a phenotype that the cell can not grow under the normal growing condition. By introducing a polynucleotide having a nucleotide sequence encoding an amino acid sequence of SLN1 isolated from budding yeast into the SLN1-deficient cell so that SLN1 is expressed (e.g. operably linked to downstream of a promoter), the cell becomes possible to grow under the normal growing condition. The "cell deficient in at least one hybrid-sensor kinase" may be obtained, for example, by deleting at least one intrinsic hybrid-sensor kinase. First, hybrid-sensor kinase will be explained below.

(Two-component regulatory system and hybrid-sensor kinase)

Two-component regulatory system is a signal transduction system which is widely used in prokaryotic organisms and, since this system is basically composed of two proteins called a sensor and a regulator, it is called two-component regulatory system. In a typical two-component regulatory system, a sensor is composed of an input region and a histidine kinase region, and a regulator is composed of a receiver region and an output region. When the input region senses an environmental stimulus, a histidine residue in an amino acid sequence in the histidine kinase region which is well conserved among organisms is phosphorylated or dephosphorylated. Herein, phosphorylation of the histidine residue is autophosphorylation utilizing ATP as a substrate. This phosphate group is transferred to an

aspartic acid residue in an amino acid sequence in the receiver region in the regulator which is well conserved among organisms, and phosphorylation and dephosphorylation of the aspartic acid residue regulates the activity of the output region in the regulator. In the case of prokaryotic organisms, the output region is a transcription regulating factor in many cases although there are exceptions, and the regulator directly controls gene expression through the aforementioned phosphoryl transfer in response to stimuli sensed by the sensor.

A sensor takes a more complicated structure in some cases unlike the aforementioned typical structure. For example, in addition to a structure composed of an input region and a histidine kinase region, following this, the sensor contains a receiver region, which is observed in a regulator, on its C-terminal side in some cases. In this case, the phosphorylay system of a phosphate group becomes more complicated, and it is known that a phosphate is transferred from the sensor to a regulator called a response regulator via an intervening protein having a transmitter region called a phosphotransmitter. That is, when the input region of the sensor senses stimuli, phospate is transferred to mediate signal transduction from a histidine residue of the histidine kinase region in the same molecule to an aspartic acid residue of the receiver region in the same molecule, then, to a histidine residue of the phosphotransmitter, finally, to an aspartic acid residue of the receiver region in

a response regulator. Like this, two-component regulatory system is associated with three proteins in some cases. Such the sensor involved in signal transduction system through phosphoryl transfer composed of three proteins and having the aforementioned structural characteristic is referred to as "hybrid-sensor kinase". Hybrid-sensor kinase is found not only in a prokaryotic organism but also in an eukaryotic microorganism such as yeast, a plant and the like, and is involved in response to a variety of stimuli or stresses.

Herein, an input region of a hybrid-sensor kinase is a region present at the N-terminal of the kinase, and have a transmembrane region in many cases. The transmembrane region can be revealed by a structure prediction analysis using a structure prediction software, for example, TMpred program [K. Hofmann & W. Stoffel, Biol. Chem. Hoppe-Seyler, 374, 166 (1993)] which is available, for example, from http://www.ch.embnet.org/software/TMPRED_form.html. A histidine kinase region of a hybrid-sensor kinase is, for example, a region following the C-terminal of the input region, and is a region characterized in that it has five conserved motifs common to general histidine kinases as described in Parkinson, J.S. & Kofoid, E.C. (1989) Annual Review of Genetics 23:311-336, Stock, J.B. et. al. (1989) Microbiological Reviews 53(4):450-490. For example, in the hybrid-sensor kinase SLN1 of budding yeast, a histidine kinase region is the region from amino acid residues

556 to 908. A receiver region of a hybrid-sensor kinase is, for example, a region following the C-terminal of the histidine kinase region, and is a region characterized in that it has three conserved motifs common to general histidine kinases as described in Parkinson, J.S. & Kofoid, E.C. Annual Review of Genetics 23:311-336(1989), Stock, J.B. et. al.(1989) Microbiological Reviews 53(4): 450-490. For example, in the hybrid-sensor kinase SLN1 of budding yeast, a receiver region is the region from amino acid residues 1088 to 1197.

As a signal transduction system after a response regulator, in addition to a simple system in which an output region of a regulator is a transcription regulating factor as described above, as a more complicated system, there is known a system in which a signal is transmitted to a transcription regulating factor participating in control of gene expression, via MAP kinase cascade which is associated with various controls in a cell.

Specific examples of a hybrid-sensor kinase and a signal transduction system which involves the hybrid-sensor kinase will be explained below.

(Hybrid-sensor kinase of budding yeast)

In budding yeast (Saccharomyces cerrevisiae), the hybrid-sensor kinase SLN1 is utilized for signal transduction relating to osmolarity response. The SLN1 is a sole histidine kinase found in budding yeast. SLN1 is an osmosensing histidine

kinase having a transmembrane region in its input region, and mediates a phosphoryl transfer signal to the response regulator SSK1 via the phosphotransmitter YPD1. Downstream of the signal transduction, MAP kinase cascade composed of three kinases SSK2(MAPKKK), PBS2(MAPKK) and HOG1(MAPK) lies to regulate expression of genes involved in osmolarity adaptation such as glycerol biosynthesis and the like. The output region of the response regulator SSK1 has an activity of phosphorylating SSK2. The SSK1 is negatively controled by phophorylation of an aspartic acid residue in its receiver region, the phosphorylating activity of whose output region is inhibited. Specifically, at a normal osmolarity, a histidine residue in the histidine kinase region of SLN1 is autophosphorylated, and the phosphate is subsequently transferred to an aspartic acid residue of the receiver region in the same molecule, then, to a histidine residue of YPD1, finally, to an aspartic acid residue in the receiver region of SSK1. By phosphorylation of an aspartic acid residue in the receiver region of SSK1, the phosphorylating activity of the output region of SSK1 is suppressed, and the phosphate is not transferred to a MAP kinase cascade composed of SSK2, PBS2 and HOG1, and then expression of genes involved in osmolarity adaptation such as glycerol biosynthesis and the like are not induced. On the other hand, under a condition of high osmolarity, since autophosphorylation of a histidine residue of the histidine kinase region is inhibited in SLN1, the MAP kinase cascade

composed of SSK2, PBS2 and HOG1 is activated, and then expression of genes involved in osmolarity adaptation such as glycerol biosynthesis and the like is induced (Maeda, T. et. al. (1994) Nature 369:242-245).

(Hybrid-sensor kinase of fission yeast)

In fission yeast (Scchizosaccharomyces pombe), three kinds of hybrid-sensor kinases PHK1 (MAK2), PHK2 (MAK3) and PHK3 (MAK1) participate in regulation of cell cycle progression [G(2) to M phase transition] and oxidative stress response. In a fission yeast, there is no histidine kinase other than PHK1, PHK2 and PHK3. PHK1 and PHK2 are histidine kinases responsive to oxidative stress such as hydrogen peroxide and the like (Buck, V. et. al., Mol. Biol. Cell 12:407-419). Three kinds of hybrid-sensor kinases PHK1, PHK2 and PHK3 metiate a phosporyl transfer signal to the response regulator MCS4 via the phosphotransmitter SPY1 (MPR1). Downstream of this signal transduction, a MAP kinase cascade composed of three kinases WAK1 (MAPKKK), WIS1 (MAPKK) and STY1 (MAPK) lies to regulate expression of genes involved in regulation of cell cycle progression and oxidative stress response. The output region of the response regulator MCS4 has an activity of phosphorylating WAK1. The MCS4 is negatively controled by phosphorylation of an aspartic acid residue in its receiver region, the phosphorylating activity of whose output region is inhibited.

Specifically, under a normal condition, each of histidine residues in the histidine kinase regions of PHK1 to PHK3 is autophosphorylated, and the phosphates are transferred to each of aspartic acid residues of receiver regions in the same molecule, then, to a histidine residue of SPY, finally, to an aspartic acid residue in the receiver region of MCS4. By phosphorylation of an aspartic acid residue in the receiver region of MCS4, the phophorylating activity of the output region of MCS4 is suppressed, and the phosphate is not transferred to a MAP kinase cascade composed of WAK1, WIS1 and STY1, and then expression of genes involved in regulation of cell cycle progression and stress response are not induced. On the other hand, under a stress condition, autophosphorylation of each of histidine residues of the histidine kinase regions in PHK1 to PHK3 is inhibited, a MAP kinase cascade composed of WAK1, WIS1 and STY1 is activated, and expression of genes involved in control of cell cycle progression and oxidative stress response are induced. As a result, it is observed such a phenotype that G(2) to M phase transition in cell cycle progression of the fission yeast is promoted, and that a dividing cell length becomes remarkably shorter than usual (Aoyama, K. et. al. (2001) Boisci. Biotechnol. Biochem. 65:2347-2352).

(Hybrid-sensor kinase of bacterium)

In a prokaryotic organism Escherichia coli, the

hybrid-sensor kinase RcsC participates in control of expression of the cps operon involved in capsular polysaccharide synthesis. RcsC is a histidine kinase having a transmembrane region, and it is known that it mediates a phosphoryl transfer signal to the response regulator RcsB via the phosphotransmitter YojN. The output region of RcsB has an activity of inducing transcription of the cps operon. Specifically, under a normal condition, a histidine residue in the histidine kinase region of RcsC is autophosphorylated, and the phosphate is transferred to an aspartic acid residue of the receiver region in the same molecule, then, to a histidine residue of YojN, finally, to an aspartic acid residue in the receiver region of RcsB. By phosphorylation of an aspartic acid residue in the receiver region of RcsB, the cps operon transcription inducing activity of the output region of RcsB is suppressed, and expression of genes involved in capsular polysaccharide synthesis are not induced. On the other hand, under a condition of high osmolarity, in RcsC, autophosphorylation of a histidine residue in the histidine kinase region is inhibited, the cps operon transcription inducing activity of the output region of RcsB is activated, and expression of genes involved in capsular polysaccharide synthesis are induced (Clarke, D.J. et. al. (2002) J. Bactriol. 184:1204-1208).

A bioluminescent marine microorganism Vibrio harveyi emits fluorescent light generated in luciferase reaction

depending on its own cell density. Hybrid-sensor kinases LuxN and LuxQ parcipite in control of expression of a gene involved in this cell density-responsive bioluminescence. LuxN and LuxQ are histidine kinases each having a transmembrane region. To sense its own cell density, V. harveyi produces and secrets two kinds of substances (AI-1, AI-2) called autoinducer. AI-1 is sensed by LuxN and AI-2 is sensed by LuxQ to convey cell-density information. LuxN and LuxQ mediate phosphoryl transfer signals to the response regulator LuxO via the phosphotransmitter LuxU. The output region of LuxO has an activity of inducing transcription of the luciferase operon. To specifically explain by referring to LuxN, when a cell density is low, since AI-1 in the environment is at low level and is not sensed by the input region of LuxN, a histidine residue in the histidine kinase region of LuxN is autophosophorylated. The phosphate is transferred to an aspartic acid residue of the receiver region in the same molecule, then, to a histidine residue of LuxU, finally, to an aspartic acid residue in the receiver region of LuxO. By phosphorylation of an aspartic acid residue in the receiver region of LuxO, the luciferase operon transcription inducing activity of the output region of LuxO is suppressed, and expression of genea involved in bioluminescence are not induced. On the other hand, under a high cell density condition, since AI-1 in environment is at high level and is sensed by the input region of LuxN, autophosphorylation of a histidine residue of

the histidine kinase region is inhibited in LuxN, the luciferase operon transcription inducing activity of the output region of LuxO is activated, and bioluminescence is induced (Freeman, J.A. et.al. (2000) Mol. Microbiol. 35:139-149).

(Hybrid-sensor kinase of plant)

In a higher plant Arabadopsis thaliana, receptor proteins CRE1, AHK2 and AHK3 for a plant hormone cytokinin are hybrid-sensor kinases. Receptor proteins CRE1, AHK2 and AHK3 are all cytokinin-sensitive histidine kinase having a transmembrane region (Inoue, T. et. al.(2001) Nature 409:1060-1063). CRE1 mediates a phosphoryl transfer signal to response regulators ARR1, ARR2 and ARR10 via phosphotransmitters AHP1 and AHP2. It is considered that output regions of ARR1, ARR2 and ARR10 have an activity of inducing transcription of cytokinin-inducing genes ARR4 to ARR7. Specifically, in the presence of cytokinin, a hisitidine residue in the histidine kinase region of CRE1 is autophosphorylated, and the phosphate is transferred to an aspartic acid residue of the receiver region in the same molecule, then, to histidine residues of AHP1 and AHP2, finally, to aspartic acid residues in receiver regions of ARR1, ARR2 and ARR10. By phosphorylation of aspartic acid residues in receiver regions of ARR1, ARR2 and ARR10, a gene transcription inducing activity of output regions of ARR1, ARR2 and ARR10 are promoted, and expression of cytokinin-responsive

genes ARR4 to 7 is induced (Hwang, I. & Sheen J.(2001) Nature 413:383-389).

(Cell deficient in at least one hybrid-sensor kinase)

"The cell deficient in at least one hybrid-sensor kinase" means a cell in which function of at least one intrinsic hybrid-sensor kinase is lost. Examples of the cell include a cell in which production of at least one intrinsic hybrid-sensor kinase is deleted, suppressed or inhibited, a cell in which activity of at least one intrinsic hybrid-sensor kinase is deleted, suppressed or inhibited, and the like. More specific examples include budding yeast deficient in SLN1, fission yeast deficient in all of three of PHK1, PHK2 and PHK3, Escherichia coli deficient in RcsC, V. harveyi deficient in LuxN, Arabidopsis thaliana deficient in CRE1, and the like.

In order to prepare the "cell deficient in at least one hybrid-sensor kinase", for example, deletion, addition, substitution or the like of one or more nucleotides are introduced into the whole or a part of a promoter region or a coding region of a gene encoding hybrid-sensor kinase to be deleted. Specifically, for example, the SLN1-deficient budding yeast strain TM182 can be prepared by the method described in Maeda, T. et. al.(1994) Nature 369:242-245, the PHK1, PHK2 and PHK3-deficient fission yeast strain KI011 can be prepared by the method described in Aoyama, K. et. al. (2001) Boisci.

Biotechnol. Biochem. 65:2347-2352. In addition, the RcsC-deficient Escherichia coli strain SRC122 can be prepared by the method described in Suzuki, T., et. al. (2001) Plant Cell Physiol. 42:107-113, and the LuxN-deficient V. harveyi strain BNL63 can be prepared by the method described in Freeman, J.A. et. al. (2000) Mol. Micobiol. 35:139-149. For preparing a CRE1-deficient Arabidopsis thaliana, for example, a clone defective in cytokine response is selected from clones obtained by mutagenesis of Arabidopsis thaliana according to the method described in Inoue, T. et. al. (2001) Nature 409:1060-1063. Genomic CRE1 gene fragment is amplified by PCR using a primer designed based on the nucleotide sequence of the genomic CRE1 gene listed in Genebank accession AB049934 and using a genomic DNA of the selected clone as a template, and its nucleotide sequence is confirmed, whereby, a CRE1-deficient clone which can not express CRE1 can be selected.

Alternatively, a cell deficient in unknown hybrid-sensor kinase besides the aforementioned kinases may be also prepared, for example, by isolating a hybrid-sensor kinase gene from a desired cell, and deleting the gene harbored by the cell by homologous recombination using the gene. For isolating a hybrid-sensor kinase gene of a desired cell, the structural characteristic of hybrid-sensor kinases can be utilized. For example, amino acid sequences around the histidine residue tobe autophosphorylated are conserved among hisitidine kinase

regions and amino acid sequences around the aspartic acid residue to which a phosphate to be transferred from the histidine residue are conserved among receiver regions. Then, a hybrid-sensor kinase gene of a desired cell can be isolated by a polymerase chain reaction (hereinafter, referred to as PCR) using an oligonucleotide designed based on a nucleotide sequence encoding the aforementioned conserved amino acid sequences as a primer, or a hybridization method using an oligonucleotide having a nucleotide sequence encoding the aforementioned conserved amino acid sequences as a prove. By examining whether or not the aforementioned structural characteristic is possessed based on an amino acid sequence deduced from a nucleotide sequence of the isolated gene, it can be confirmed that the isolated gene is a gene having a nucleotide sequence encoding an amino acid sequence of a hybrid-sensor kinase. A specific example is a PCR method described in Srilantha, T. et. al. (1998) Microbiology 144:2715-2729. For PCR and hybridization, for example, the experimental conditions using upon isolation of the "polynucleotide having a nucleotide sequence encoding an amino acid sequence of osmosensing hisitidine kinase having no transmembrane region" described later may be used.

Alternatively, a hybrid-sensor kinase gene may be also isolated using, as an index, the functional complementation in budding yeast in which expression of SLN1 is conditionally suppressed, for example, according to the method described in

Nagahashi, S. et. al. (1998) Microbiology 144:425-432.

(Osmosensing histidine kinase having no transmembrane region)

Then, the "osmosensing histidine kinase having no transmembrane region" to be introduced into the aforementioned "cell deficient in at least one hybrid-sensor kinase" in a functional form will be explained.

In filamentous fungus, a histidine kinase having a structure similar to that of the aforementioned hybrid-sensor kinase is isolated. The histidine kinase has a histidine kinase region and a receiver region which are observed in hybrid-sensor kinases, and has no transmembrane region, which is observed in many hybrid-sensor kinases, in its input region, and further has a characteristic structure in which amino acid sequences composed of about 90 amino acids having the amino acid sequence homology to each other are present repeatedly about six times, in place of the transmembrane region. Although a signal transduction pathway from this histidine kinase has not been completely clarified, it is known that the signal transduction participates in osmolarity response.

In the present invention, "homology" refers to identity of sequences between two genes or two proteins. The "homology" is determined by comparing two sequences aligned in the optimal state, over a region of a sequence of a subject to be compared. Herein, in optimal alignment of nucleotide sequences or amino

acid sequences to be compared, addition or deletion (e.g. gap etc.) may be allowable. Such the "homology" can be calculated by homology analysis with making alignment using a program of FASTA [Pearson & Lipman, Proc. Natl. Acad. Sci. USA, 4, 2444-2448(1998)], BLAST [Altschul et. al. Journal of Molecular Biology, 215, 403-410(1990)], CLUSTAL W [Thompson, Higgins & Gibson, Nucleic Acid Research, 22, 4673-4680(1994a)] and the like. The above programs are available to the public, for example, in homepage (http://www.ddbj.nig.ac.jp) of DNA Data Bank of Japan [international DNA Data Bank managed in Center for Information Biology and DNA Data Bank of Japan (CIB/DDBJ)). Alternatively, the "homology" may be also obtained by using commercially available sequence analysis software. Specifically, the homology can be calculated, for example, by performing homology analysis with making alignment by the Lipman-Pearson method [Lipman, D. J. and Pearson, W. R., Science, 227, 1435-1441,(1985)] using GENETYX-WIN Ver.5 (manufactured by Software Development Co., Ltd.).

Herein, as the "structure in which amino acid sequences composed of about 90 amino acids having the amino acid sequence homology to each other are repeatedly present about six times", for example, there is a repeat sequence region described in Alex,L.A. et. al. (1996) Proc. Natl. Acad. Sci. USA 93:3416-3421, Ochiai, N. et. al. (2001) Pest Manag. Sci. 57:437-442, Oshima, M. et. al. (2002) Phytopathology 92:75-80 and the like, and such

the structure is present at the N-terminal region of the hisitide kinase. The "amino acid sequences composed of about 90 amino acids are repeatedly present about six times" include an amino acid sequence motif composed of about 90 amino acids is repeated five times followed by a sixth truncated repeat sequence (5.7 times repeat), an amino acid sequence motif composed of about 90 amino acids is repeated six times followed by a seventh truncated repeat sequence (6.7 times repeat), and the like. Specifically, in amino acid sequence of a histidine kinase of the present invention, examples of the "a region in which amino acid sequences composed of about 90 amino acids having the amino acid sequence homology to each other are present repeatedly about six times" include a region from amino acid residues 190 to 707 in an amino acid sequence represented by SEQ ID NO: 1 (5.7 times repeat), a region from amino acid residues 189 to 706 in an amino acid sequence represented by SEQ ID NO: 16 (5.7 times repeat), a region from amino acid residues 176 to 693 in an amino acid sequence represented by SEQ ID NO: 41 (5.7 times repeat), a region from amino acid residues 192 to 709 in an amino acid sequence represented by SEQ ID NO: 55 (5.7 times repeat), and a region from amino acid residues 299 to 911 in an amino acid sequence represented by SEQ ID NO: 68 (6.7 times repeat), and the like.

The "osmosensing histidine kinase having no transmembrane region" is the aforementioned histidine kinase characteristic in filamentous fungus, and refers to a osmosensing protein having

a repeat sequence region of amino acid sequences composed of about 90 amino acids having the amino acid sequence homology to each other, a histidine kinase region and a receiver region, and having no transmembrane region.

In order to confirm that a protein has the function of osmosensing histidine kinase, enhancement of the sensitivity of a cell to osmolarity stress may be confirmed when the protein (histidine kinase) is deleted from the cell. Alternatively, it may be also confirmed that a protein (histidine kinase) is osmosensing histidine kinase, by confirming that expression of the protein in an osmosensing hybrid-sensor kinase SLN1-deficient budding yeast cell results in a functional complementation of the SLN1 and the budding yeast cell capable of growing.

Among filamentous fungi, mainly, in Neurospora crassa which is a model organism of filamentous fungus, a plant pathogenic filamentous fungus which is a pathogenic microorganism, a host of which is a plant, or the like, the presence of the "osmosensing histidine kinase having no transmembrane region" is reported.

Examples of the "osmosensing histidine kinase having no transmembrane region" of the present invention include an osmosensing histidine kinase having no transmembrane region, which has an amino acid sequence selected from the group consisting of:

(a) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which has an amino acid sequence homology of 95% or more to the amino acid sequence represented by any of SEQ ID NOs: 41, 55 and 68;

(b) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Fusarium oxysporum-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 52 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 53 as primers;

(c) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Mycospharella tritici-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 64 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 65 as primers;

(d) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Thanapethorus cucumeris-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 85 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 86 as primers;

(e) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is derived from Phytophthora infestans and has the amino acid sequence represented by SEQ ID NO: 90;

(f) the amino acid sequence represented by SEQ ID NO: 41;

(g) the amino acid sequence represented by SEQ ID NO: 55; and

(h) the amino acid sequence represented by SEQ ID NO: 68.

A preferred amino acid sequence homology in the above (a) may for example be about 95%, or higher such as about 98%. The difference from the amino acid sequence represented by any of SEQ ID : 41, 55 and 68 observed in the amino acid sequence of the above (a) may for example be a variation such as the deletion, substitution and addition of amino acids. Such a variation includes a variation which can artificially be introduced by means of a site-directed mutagenesis method or a mutagenic treatment as well as a polymorphic variation which occurs naturally such as a difference in an amino acid sequence resulting from the difference by the species or strains from which the protein is derived. As the site-directed mutagenesis method, for example, there is mentioned the method which utilizes amber mutations (capped duplex method, Nucleic Acids Res., 12, 9441-9456 (1984)), the method by PCR utilizing primers for introducing a mutation and the like.

At least one, specifically one to several (herein "several" means about 2 to about 10), or more amino acid residues may be

varied in the above variations. The amino acid residues may be varied in any numbers as far as the effect of the present invention can be observed.

Of the deletion, addition, and substitution, the substitution is particularly preferred in the amino acid variation. Amino acids that are similar to each other in hydrophobicity, charge, pK, stereo-structural characteristic, or the like are more preferably replaced with each other. For example, such substitutable amino acids are in each of the following groups: 1) glycine and alanine; 2) valine, isoleucine, and leucine; 3) aspartic acid, glutamic acid, asparagine, and glutamine; 4) serine and threonine; 5) lysine and arginine; and 6) phenylalanine and tyrosine.

The "osmosensing histidine kinase having no transmembrane region" will be further explained with the specific examples shown below.

(Osmosensing histidine kinase having no transmembrane region of Neurospora crassa)

A protein OS-1 encoded by an os-1 gene isolated from an osmosensing mutant os-1 of Neurospora crassa can be mentioned as the "osmosensing histidine kinase having no transmembrane region" (Schumacher, M. M. et. al. (1997) Current Microbiol. 34:340-347, Alex, L. A. et. al. (1996) Proc. Natl. Acad. Sci. USA 93:3416-3421). Amino acid sequences of OS-1 and nucleotide

sequences of the os-1 gene are published (amino acid sequence: Genebank accession AAB03698, AAB01979, nucleotide sequence: Genebank accession U50263, U53189), and utility of OS-1 and os-1 gene in screening system for antifungal compounds is described in US 5, 939, 306. Since Neurospora crassa mutant os-1 has the higher sensitivity to high osmolarity stress than that of a wild strain, it has been found that OS-1 is an osmosensing histidine kinase involved in osmolarity adaptation in Neurospora crassa. It is known that OS-1 has the aforementioned structural characteristic based on its amino acid sequence. In addition, it is known that Neurospora crassa mutant os-1 has the resistance to fungicides containing, as an active ingredient, a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" or a phenylpyrrole antifungal compound. Further, a gene mutation which leads to an amino acid substitution in a characteristic repeat sequence region of OS-1 was observed in the os-1 mutant gene isolated from Neurospora crassa mutant exhibiting the resistance to a fungicide containing a dicarboxyimide antifungal compound as an active ingredient (Miller, T. K. et. al. (2002) Fungl Gen. Biol. 35:147-155). From the foregoing, it is predicted that an antifungal compound contained as an effective ingredient in the aforementioned fungicide targets OS-1 of Neurospora crassa.

(Osmosensing histidine kinase having no transmembrane region

of Botryotinia fuckeliana)

Examples of the "osmosensing histidine kinase having no transmembrane region" include BcOS-1 of Botryotinia fuckeliana. The BcOS-1 gene was isolated as a gene homologous to Neurospora crassa OS-1 gene, and nucleotide sequencez and amino acid sequences are published (nucleotide sequence: GeneBank accession AF396287, AF435964, amino acid sequence: GeneBank accession AAL37947, AAL30826). It is known that BcOS-1 has the aforementioned structural characteristic based on its amino acid sequence. In addition, in the BcOS-1 gene isolated from a Botryotinia fuckeliana strain resistant to a fungicide containing a dicarboxyimide antifungal compound as an active ingredient, a mutation which leads to amino acid substitution in the characteristic repeat sequence region of BcOS-1 was observed, as in the OS-1 gene isolated from a Neurospora crassa strain resistant to a fungicide containing a dicarboxyimide antifungal compound as an active ingredient. Further, since an antifungal compound-resistant mutant deficient in the BcOS-1 has the higher osmolarity sensitivity than that of a wild strain, it is known that BcOS-1 is osmosensing histidine kinase (Oshima, M. et. al. (2002) Phypotathology 92:75-80).

More specifically, examples of BcOS-1 include BcOS-1 having an amino acid sequence represented by SEQ ID NO: 1 which was isolated from Bc-16 strain described in Example.

(Osmosensing histidine kinase having no transmembrane region of Magnaporthe grisea)

Example of the "osmosensing histidine kinase having no transmembrane region" include HIK1 of Magnaporthe grisea. The hik1 gene is a gene homologous to Neurospora crass os-1 gene, and a nucleotide sequence and an amino acid sequence are published (nucleotide sequence: Genebank accession AB041647, amino acid sequence: GeneBank accession BAB40947). It is known that HIK1 has the aforementioned structural characteristics such as lack of the transmembrane region based on its amino acid sequence. In addition, it is observed that Magnaporthe grisea deficient in the hik1 gene has the higher osmolarity sensitivity than that of a wild strain, demonstrating that HIK1 is an osmosensing histidine kinase (hppt://www.sci.saitama-u.ac.jp/seitai/iden/Japanese/AbstSymp3.html).

More specifically, examples of HIK1 include HIK1 having an amino acid sequence represented by SEQ ID NO: 16 which was isolated from the P-37 strain described in Example.

(Definition of filamentous fungus and yeast)

In the present invention, the "filamentous fungus" means fungi other than fungi which can be classified as yeast, among fungi consisting of Myxomycota and Eumycota, described in "Revised Edition, Classification and Identification of

Microorganisms (Volume 1), edited by Takeharu HASEGAWA, Society Publishing Center, 1984 (ISBN 4-7622-7399-6)". Examples of filamentous fungus classified in Myxomycota include Plasmodiophora brassicae belonging to Plasmodiophoromycetes. In addition, examples of filamentous fungus which is classified in Eumycota include Phytophthora infestans belonging to Mastigomycotina, Rhizopus stolonifer and Rhizopus oryzae belonging toZygomycotina, Neurospora crassa, Mycospharella tritici, Erysiphe graminis, Linocarpon cariceti, Cochliobolus miyabeanus, Botrytinia fuckeliana and Magnaporthe grisea belonging toAscomycotina, Ustilago maydis, Puccinia recondite and Thanatephorus cucumeris belonging toBasidiomycotina, Cladosporium fulvum, Alternalia kikuchiana and Fusarium oxysporum belonging to Deuteromycotina, and the like.

In addition, yeast means fungi in which they are grown mainly by budding, a single cell generation is long, a colony formed by growth of a single cell does not become hairy, but becomes white bright paste-like" as described in "Revised Edition, Classification and Identification of Microorganisms (Volume 1), edited by Takeharu HASEGAWA, Society Publishing Center, 1984 (ISBN 4-7622-7399-6)". Examples thereof include Saccharomyces cerevisiae belonging to genus Saccharomyces, Schizosaccharomyces pombe belonging to genus Schizosaccharomyces, Phichia burtonii belonging to genus Phichia, Candida albicans belonging to genus Candida, and the

like.

(Osmosensing histidine kinase having mutation which confers resistance to any of dicarboxyimide antifungal compound, aromatic hydrocarbon antifungal compound and phenylpyrrole antifungal compound, and having no transmembrane region)

As a specific example of the "osmosensing histidine kinase having no transmembrane region", there can also be exemplified "osmosensing histidine kinase having no transmembrane region" having mutation which confers resistance to any of a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" and a phenylpyrrole antifungal compound. Specifically, there can be exemplified BcOS-1 having an amino acid sequence represented by SEQ ID NO: 13 which is described in Example.

Herein, the dicarboxyimide antifungal compound is a generic name of antifungal compounds having dicarboxyimide as a basic structure, and examples thereof include antifungal compounds described in Modern Selective Fungicide-Properties, Applications, Mechanism of Action-2nd revised and enlarged edition Lyr, H. ed. Gustav Fisher Verlag, New York, USA ISBN 3-334-60455-1 Chapter 6, p99-118. Specifically, there are a compound having a structure represented by the chemical formula (1) (Procymidone: hereinafter, referred to as Compound (1) in some cases), a compound having a structure represented by the

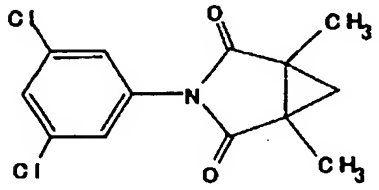
chemical formula (2) (Iprodione: hereinafter, referred to as Compound (2) in some cases), a compound having a structure represented by the chemical formula (3) (Vinclozolin: hereinafter, referred to as Compound (3) in some cases) and the like. The "aromatic hydrocarbon antifungal compound" is a generic name of antifungal compounds having a benzene ring as a basic structure, and examples thereof include antifungal compounds described in Modern Selective Fungicide-Properties, Applications, Mechanism of Action-2nd revised and enlarged edition Lyr, H. ed. Gustav Fisher Verlag, New York, USA ISBN 3-334-60455-1 Chapter 5, p75-98. Specifically, there are a compound having a structure represented by the chemical formula (4) (Quintozene: hereinafter, referred to as Compound (4) in some cases), a compound having a structure represented by the chemical formula (5) (Tolclofos-methyl: hereinafter, referred to as Compound (5) in some cases). In addition, the phenylpyrrole antifungal compound is a generic name of antifungal compounds having phenylpyrrole as a basic structure, and examples thereof include antifungal compounds described in Modern Selective Fungicide-Properties, Applications, Mechanism of Action-2nd revised and enlarged edition Lyr, H. ed. Gustav Fisher Verlag, New York, USA ISBN 3-334-60455-1 Chapter 19, p405-407. Specifically, there are a compound having a structure represented by the chemical formula (6) (Fludioxonil: hereinafter, referred to as Compound (6) in some cases), a compound having a structure

represented by the chemical formula (7) (Fenpiclonil: hereinafter, referred to as Compound (7) in some cases) and the like.

Chemical formulas of the aforementioned dicarboxyimide antifungal compound, "aromatic hydrocarbon antifungal compound" and phenylpyrrole antifungal compounds are shown below.

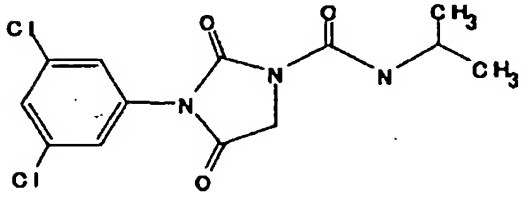
(1) Compound having a structure represented by the chemical formula (1) (Compound (1))

Chemical formula (1)



(2) Compound having a structure represented by the chemical formula (2) (Compound (2))

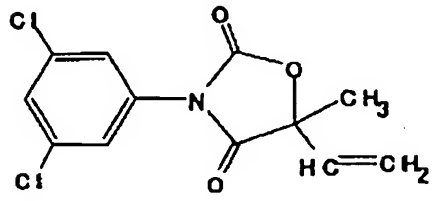
Chemical formula (2)



(3) Compound having a structure represented by the chemical

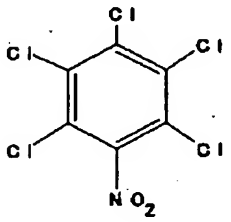
formula (3) (Compound (3))

Chemical formula (3)



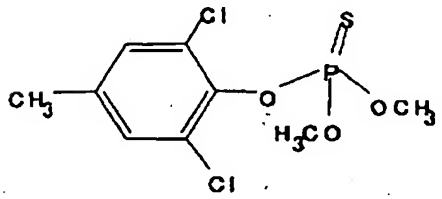
(4) Compound having a structure represented by the chemical formula (4) (Compound (4))

Chemical formula (4)



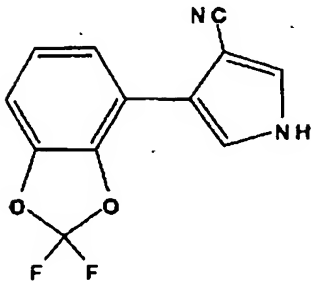
(5) Compound having a structure represented by the chemical formula (5) (Compound (5))

Chemical formula (5)



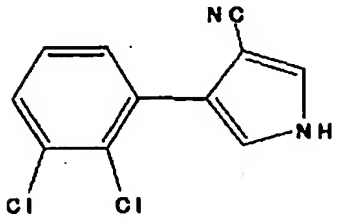
(6) Compound having a structure represented by the chemical formula (6) (Compound (6))

Chemical formula (6)



(7) Compound having a structure represented by the chemical formula (7) (Compound (7))

Chemical formula (7)



The "mutation which confers resistance to any of a dicarboxyimide antifungal compound, an aromatic hydrocarbon antifungal compound and a phenylpyrrole antifungal compound" indicates a mutation which can be found in the "osmosensing histidine kinase having no transmembrane region" produced by a filamentous fungus mutant having resistance to any of a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" and a phenylpyrrole antifungal compound, that is, substitution, addition or deletion of one or more amino acids which confer resistance to a dicarboxyimide antifungal

compound, an "aromatic hydrocarbon antifungal compound" and a phenylpyrrole antifungal compound, provided that mutation by which the "osmosensing histidine kinase having no transmembrane region" becomes not to function as histidine kinase is eliminated. Herein, a mutant of filamentous fungus having resistance to any of a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" and a phenylpyrrole antifungal compound may be filamentous fungus isolated from the nature to which any of a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" and a phenylpyrrole antifungal compound was applied, or may be resistance-acquired filamentous fungus selected by artificially culturing filamentous fungus in the presence of a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" or phenylpyrrole antifungal compound.

Specifically, in BcOS-1 in the "osmosensing histidine kinase having no transmembrane region" of Botryotinia fuckeliana, amino acid-substitution I365S which confers resistance to a dicarboxyimide antifungal compound is reported in Oshima, M. et al. (2002) Phytopathology 92:75-80 (herein, "I365S" means that isoleucine at amino acid residue 365 is substituted with serine. Hereinafter, amino acid substitution is described similarly). As an amino acid substitution which confers resistance to a dicarboxyimide antifungal compound in OS-1 which is the "osmosensing histidine kinase having no transmembrane region"

of Neuorspora crassa, T368P, Q388S, E418E, L459M, A578V, G580R, I582M, M639V, A578V, G580G and L625P are reported and, as an amino acid deletion, 680K is reported in Miller, T.K. et al. (2002) Fungal Gen. Biol. 35:147-155 (hereinafter, 680K means that lysine at amino acid residue 680 is deleted. Hereinafter, amino acid deletion is described similarly). In addition, amino acid substitution which confers resistance to a phenylpyrrole antifungal compound in the OS-1 of Neurospora crassa, A578V, G580R and L625P are reported in Ochiai, N. et al. (2001) Pest Management Sci. 57:437-442.

Besides the aforementioned resistance mutation, resistance mutation may be found by analyzing an amino acid sequence of the "osmosensing hystidine kinase having no transmembrane region" isolated from a mutant filamentous fungus having resistance to any of a dicarboxyimide antifungal compound, an "aromatic hydrocarbon antifungal compound" and a phenypyrrole antifungal compound, and comparing with an amino acid sequence of the protein in a sensitive wild strain.

(Preparation of transformed cell in which a polynucleotide having a nucleotide sequence encoding an amino acid sequence of osmosensing histidine kinase having no transmembrane region is introduced in a functional form into a cell deficient in at least one hybrid-sensor kinase)

The transformed cell in which a polynucleotide having a

nucleotide sequence encoding an amino acid sequence of osmosensing histidine kinase having no transmembrane region (hereinafter, referred to as present histidine kinase in some cases) is introduced in functional form, can be obtained by introducing a "polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present hystidine kinase" or the like into a "cell deficient in at least one hybrid-sensor kinase" which is to be a host cell, as described below.

Examples of the "polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present hystidine kinase" include a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present hystidine kinase which is derived from a plant-pathogenic filamentous fungus, more specifically, for example, a polynucleotide having a nucleotide sequence encoding an amino acid sequence selected from the group consisting of:

(a) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which has an amino acid sequence homology of 95% or more to the amino acid sequence represented by any of SEQ ID NOs: 41, 55 and 68;

(b) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Fusarium oxysporum-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 52 and

an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 53 as primers;

(c) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Mycospharella tritici-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 64 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 65 as primers;

(d) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is encoded by a DNA amplified by a polymerase chain reaction using a Thanapethorus cucumeris-derived cDNA as a template and using an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 85 and an oligonucleotide having the nucleotide sequence represented by SEQ ID NO: 86 as primers;

(e) an amino acid sequence of an osmosensing histidine kinase having no transmembrane region, which is derived from Phytophthora infestans and has the amino acid sequence represented by SEQ ID NO: 90;

(f) the amino acid sequence represented by SEQ ID NO: 41;

(g) the amino acid sequence represented by SEQ ID NO: 55; and

(h) the amino acid sequence represented by SEQ ID NO: 68.

One example of a process for producing the transformed cell will be shown below.

(1) Preparation of cDNA

First, total RNA is prepared from filamentous fungus, for example, according to the method described in Molecular Cloning 2nd edition authored by J., Sambrook, E., F., Frisch, T., Maniatis. Specifically, for example, a part of a fungal tissue is collected from Neurospora crassa, Botrytinia fuckeliana, Magnaporthe grisea, Phytophthora infestans, Thanatephorus cucumeris, Fusarium oxysporum, Mycospharella tritici, Thanatephorus cucumeris, Thanatephorus cucumeris and the like, the collected tissue is frozen in liquid nitrogen, and is physically ground with a mortar or the like. Then, total RNA may be prepared by the conventional method such as (a) a method of adding a solution containing guanidine hydrochloride and phenol or a solution containing SDS and phenol to the resulting ground material, to obtain total RNA, or (b) a method of adding a solution containing guanidine thiocyanate to the aforementioned ground material, and further adding CsCl, followed by centrifugation, to obtain total RNA. In the procedures, a commercially available kit such as RNeasy Plant Mini Kit (manufactured by QIAGEN) may be also used.

Then, the thus prepared total RNA is used to prepare a cDNA. For example, cDNA may be prepared by reacting a reverse transcriptase on the total RNA after an oligo-dT chain or a random primer is annealed to total RNA. In addition, further, a double-stranded cDNA can be prepared, for example, by reacting

RNaseH, DNA Polymerase I on said cDNA. In the procedures, a commercially available kit such as SMARTTM PCR cDNA Synthesis Kit (manufactured by Clonech), cDNA Synthesis Kit (manufactured by TAKARA SHUZO Co.,Ltd.), cDNA Synthesis Kit (manufactured by Amersham Pharmacia) and ZAP-cDNA Synthesis Kit (manufactured by Stratagene) can be used.

(2) Cloning

When a nucleotide sequence of a desired present histidine kinase is known, a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase can be obtained, for example, from the cDNA prepared as described above, for example, by PCR using as a primer an oligonucleotide having a partial nucleotide sequence of the known nucleotide sequence, or a hybridization method using as a probe an oligonucleotide having a partial nucleotide sequence of the known nucleotide sequence.

A polynucleotide having a nucleotide sequence encoding an amino acid sequence of BcOS-1 which is the present histidin kinase can be prepared from a cDNA of Botryotinia fuckeliana, for example, by PCR using as a primer an oligonucleotide having a partial nucleotide sequence of the nucleotide sequence represented by SEQ ID NO: 2, or a hybridization method using as a probe an oligonucleotide having a partial nucleotide sequence of the nucleotide sequence represented by SEQ ID NO: 2.

In addition, a polynucleotide having a nucleotide sequence encoding an amino acid sequence of HIK1 which is the present histidine kinase can be obtained from a cDNA of Magnaporthe grisea, for example, by PCR using as a primer an oligonucleotide having a partial nucleotide sequence of the nucleotide sequence represented by SEQ ID NO: 17, or hybridization method using as a probe an oligonucleotide having a partial nucleotide sequence of the nucleotide sequence represented by SEQ ID NO: 17.

When a nucleotide sequence of a desired present histidine kinase is unknown, a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase can be obtained by a hybridization method using as a probe an oligonucleotide having a partial nucleotide sequence of the nucleotide sequence of the present histidine kinase, the nucleotide sequence of which is known, or by PCR using as a primer an oligonucleotide designed based on a highly homologous amino acid sequence in plural present histidine kinases, an amino acid sequence of which is known. As the highly homologous amino acid sequence among plural present histidine kinases, amino acid sequences of which are known, for example, there can be exemplified amino acid sequences of a conserved motifs observed in the "repeat sequence region", the "histidine kinase region", the "receiver region" and the like, characterized in the structure of the present histidine kinase.

More specifically, when the BcOS-1 gene of Botryotinia

fuckeliana is obtained by PCR, for example, oligonucleotides designed and synthesized based on nucleotide sequences of about 20bp to about 40bp which are selected from a 5′ non-translated region and a 3′ non-translated region, respectively, of the nucleotide sequence represented by SEQ ID NO: 2 can be used as a primer set. Examples of the primer set include a set of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 3 and an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 4. A PCR reaction solution to be used may be prepared by adding a reaction solution designated by a commercially available DNA polymerase or kit as described below to 250ng of a cDNA. The PCR reaction conditions can be appropriately changed depending on a primer set to be used, and examples thereof include the condition of maintaining a temperature at 94°C for 2 minutes, then maintaining a temperature at about 8°C for 3 minutes and, thereafter, repeating around 40 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 30 seconds, then at 55°C for 30 seconds, then at 72°C for 4 minutes, and the condition of repeating 5 to 10 cycles of incubation, each cycle comprising maintaining a temperature at 94 °C for 5 seconds, then at 72°C for 4 minutes, and further repeating about 20 to 40 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then at 70°C for 4 minutes. For the procedures, commercially available DNA polymerases contained in Heraculase™

Enhanced DNA Polymerase(manufactured by Toyobo Co.,Ltd.), Advantage cDNA PCR Kit (manufactured by Clonetech), and commercially available kits such as TAKARA Ex Taq (manufactured by TAKARA SHUZO Co.,Ltd.), PLATINUMTM PCR SUPER Mix (manufactured by Lifetech Oriental), KOD-Plus-(manufactured by Toyobo Co.,Ltd.)and the like can be used.

When the hik1 gene of Magnaporthe grisea is obtained by PCR, for example, oligonucleotides designed and synthesized based on nucleotide sequences selected from a 5′ non-translation region and a 3′ non-translation region, respectively, of the nucleotide sequence represented by SEQ ID NO: 17 can be used as a primer set. Examples of the primer set include a set of an oligonucleotide comprising the nucleotide sequence represented by SEQ ID NO: 18 and an oligonucleotide comprising the nucleotide sequence represented by SEQ ID NO: 19. A PCR reaction solution and the reaction conditions as described above can be used to perform PCR, to obtain the hik1 gene.

When a gene of the present histidine kinase, a nucleotide sequence of which is not known, is obtained from Fusarium oxysporum, Mycospharella tritici, Thanatephorus cucumeris or Phytophthora infestans, a polynucleotide having a nucleotide sequence encoding a part of an amino acid sequence of the present histidine kinase (hereinafter, referred to as present gene fragment in some cases) can be obtained by the following PCR. As a primer set, for example, a set of oligonucleotides designed

and synthesized based on amino acid sequences of a conserved motifs observed in the "repeat sequence region", the "histidine kinase region", the "receiver region" and the like, characterized in the structure of the present histidine kinase, can be used. Examples of the primer set include a primer set of an oligonucleotide having the nucleotide sequence represented by any of SEQ ID NOs: 30 to 34 and an oligonucleotide having the nucleotide sequence represented by any of SEQ ID NOs: 35 to 40.

Specifically, in the case of Fusarium oxysporum, for example, using an oligonucleotide primer having the nucleotide sequence represented by SEQ ID NO: 33 and an oligonucelotide primer having the nucleotide sequence represented by SEQ ID NO: 38, and using KOD-Plus-(TOYOBO), amplification is performed under the conditions in which a temperature is maintained at 94°C for 2 minutes, and then 35 cycles of incubation are repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds and, further, at 68°C for 5 minutes. In addition, in the case of Mycospharella tritici, for example, using an oligonucleotide primer having the nucleotide sequence represented by SEQ ID NO: 31 and an oligonucelotide primer having the nucleotide sequence represented by SEQ ID NO: 40, and using KOD-Plus- (TOYOBO), amplification is performed under the conditions in which a temperature is maintained at 94°C for 2 minutes, and 35 cycles of incubation are repeated, each cycle comprising maintaining

a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds and, further, at 68°C for 3 minutes. In addition, in the case of Thanatephorus cucumeris, for example, using an oligonucelotide primer having the nucleotide sequence represented by SEQ ID NO: 30 and an oligonucleotide primer having the nucleotide sequence represented by SEQ ID NO: 37, and using KOD-Plus-(TOYOBO), amplification is performed under the conditions in which a temperature is maintained at 94°C for 2 minutes, and 35 cycles of incubation are repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds, further, at 68°C for 1 minute. In addition, in the case of Phytophthora infestans, for example, using an oligonucleotide primer having the nucleotide sequence represented by SEQ ID NO: 31 and an oligonucelotide primer having the nucleotide sequence represented by SEQ ID NO: 37, and using KOD-Plus-(TOYOBO), amplification is performed under the conditions in which a temperature is maintained at 94°C for 2 minutes, and 35 cycles of incubation are repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds and, further, at 68°C for 1 minute. By such the PCR, a polynucleotide having a nucleotide sequence encoding a part of an amino acid sequence of the present hisitidine kinase is amplified. A polynucleotide having a nucleotide sequence encoding a full length amino acid sequence of the present histidine kinase can be obtained by RACE method by using, for

example, SMART RACE cDNA Amplification Kit (CLONTECH) and primers designed based on a nucleotide sequence of the amplified polynucleotide (present gene fragment).

When the polynucleotide obtained as described above has revealed a nucleotide sequence encoding a full length amino acid sequence of the present histidine kinase, by PCR using an oligonucelotide having a partial nucleotide sequence of the sequence as a primer, a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase may be also obtained.

Specifically, when a gene of the present histidine kinase of Fusarium oxysporum (hereinafter, referred to FoOS-1 gene in some cases) is obtained by PCR, for example, oligonucelotides designed and synthesized based on nucleotide sequences selected from a 5'-terminal region and a 3'-terminal region, respectively, of the nucleotide sequence represented by SEQ ID NO: 42 can be used as a primer set. Examples of the primer set include a set of an oligonucleotide comprising the nucleotide sequence represented by SEQ ID NO: 52 and an oligonucelotide comprising the nucleotide sequence represented by SEQ ID NO: 53. A PCR reaction solution and the reaction conditions as described above are used to perform PCR, whereby, a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase derived from Fusarium oxysporum can be obtained.

In addition, when a gene of the present histidine kinase of Mycospharella tritici (hereinafter, referred to StOS-1 gene in some cases) is obtained by PCR, for example, oligonucleotides designed and synthesized based on nucleotide sequences selected from a 5'-teminal region and a 3'-terminal region, repectively, of the nucleotide sequence represented by SEQ ID NO: 56 can be used as a primer set. Examples of the primer set include a set of an oligonuceleotide comprising the nucleotide sequence represented by SEQ ID NO .64 and an oligonucleotide comprising the nucleotide sequence represented by SEQ ID NO: 65. A PCR reaction solution and the reaction conditions as described above are used to perform PCR, whereby, a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase derived from Mycospharella tritici can be obtained.

In addition, when a gene of the present histidine kinase of Thanatephorus cucumeris (hereinafter, referred to RsOS-1 gene in some cases) is obtained by PCR, for example, oligonucelotides designed and synthesized based on nucleotide sequences selected from a 5'-terminal region and a 3'-terminal region, respectively, of the nucleotide sequence represented by SEQ ID NO: 69 can be used as a primer set. Examples of the primer set include a set of an oligonucelotide comprising the nucleotide sequence represented by SEQ ID NO: 85 and an oligonucleotide comprising the nucleotide sequence represented by SEQ ID NO: 86. A PCR

reaction solution and the reaction conditions as described above are used to perform PCR, whereby, a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase derived from Thanatephorus cucumeris can be obtained.

When a hybridization method is used, cloning can be performed, for example, according to the method described in Molecular Cloning 2nd edition, authored by J., Sambrook, E., F., Frisch, T., Maniatis.

A probe used to obtain a gene of the present histidine kinase can be obtained by synthesizing a DNA (around about 200 bases to about 500 bases in length) having a partial nucleotide sequence of the nucleotide sequence represented by SEQ ID NO: 2, followed by radioisotope-labeling or fluorescently labeling the DNA according to the conventional method. In such the labeling of a DNA, commercially available kits such as Random Primed DNA Labelling Kit (manufactured by Boehringer), Random Primer DNA Labelling Kit Ver.2 (manufactured by TAKARA SHUZO Co.,Ltd.), ECL Direct Nucleic acid Labelling and Detection System (manufactured by Amersham Pharmacia), Megaprime DNA-labelling system (manufactured by Amersham Pharmacia) and the like may be utilized. The thus obtained probe can be used for cloning a gene of the histidine kinase such as the BcOS1-gene of Botrytinia fuckeliana, a nucleotide sequence of which is known, or a gene of the present histidine kinase, a nucleotide sequence of which

is unknown.

Examples of the hybridization condition include the stringent condition, specifically, the condition under which, in the presence of 6×SSC (0.9 M NaCl, 0.09 M trisodium citrate), 5×Denhart's solution (0.1%(w/v) Ficoll 400, 0.1%(w/v) polyvinylpyrrolidone, 0.1%BSA), 0.5% (w/v) SDS and 100 μg/ml denatured salmon sperm DNA, or in DIG EASY Hyb solution (Boehringer Manheim) containing 100 μg/ml denatured salmon sperm DNA, a temperature is maintained at 65°C, then a temperature is maintained at room temperature for 15 minutes twice in the presence of 1×SSC (0.15 M NaCl, 0.015 M trisodium citrate) and 0.5%SDS, further, a temperature is maintained at 68°C for 30 minutes in the presence of 0.1×SSC (0.015 M NaCl, 0.0015M trisodium citrate) and 0.5%SDS.

Specifically, for example, for obtaining the BcOS-1 gene of Botrytinia fuckeliana, PCR is performed by using a Botrytinia fuckeliana cDNA library phage solution (about 1,000,000 pfu) as a template, and using TAKARA LA taq™ (manufactured by TAKARA SHUZO Co.,Ltd.), and using an oligonucleotide comprising the nucleotide sequence represented by SEQ ID NO: 9 and an oligonucleotide comprising a nucleotide sequence complementary to the nucleotide sequence represented by SEQ ID NO: 10 as a primer set, whereby, a DNA for a probe is amplified, which may be collected. A PCR reaction solution to be used may be prepared by adding a reaction solution designated by a kit as described

above to 250ng of a DNA library. Examples of the PCR reaction condition include the condition under which amplification is performed by maintaining a temperature at 94°C for 2 minutes, then at 8°C for 3 minutes, and repeating 40 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 30 seconds, then, at 55°C for 30 seconds and, then, at 68°C for 5 minutes. Then, a probe labeled with ^{32}P can be prepared by using the amplified and obtained DNA as a template, and using Megaprime DNA-labelling system (Amersham Pharmacia) and using a reaction solution designated by the kit. The thus prepared probe is used to perform colony hybridization according to the conventional method, in which a temperature is maintained at 65°C in the presence of 6×SSC (0.9 M NaCl, 0.09M trisodium citrate, 5×Denharp's solution (0.1%(w/v) Ficoll 400, 0.1%(w/v) polyvinylpyrrolidone, 0.1%BSA), 0.5%(w/v)SDS and 100 μg/ml denatured Salmon sperm DNA, or in DIG EASY Hyb solution (Boehringer Mannheim), containing 100 μg/ml denated Salmon sperm DNA, then, a temperature is maintained at room temperature for 15 minutes twice in the presence of 1×SSC (0.15 M NaCl, 0.015M trisodium citrate) and 0.5%SDS and, further, a temperature is maintained at 68°C for 30 minutes in the presence of 0.1×SSC (0.015 M NaCl, 0.0015 M sodium citrate) and 0.5%SDS, whereby, a clone which hybridizes with the probe can be obtained.

In addition, a gene of the present histidine kinase having a known nucleotide sequence may be also prepared by performing

chemical synthesis of a nucleic acid, for example, according to the conventional method such as a phosphite triester method (Hunkapiller, M. et al, Nature 310, 105, 1984), based on the known nucleotide sequence.

The thus obtained polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase may be cloned into a vector according to the conventional method described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBNO-471-50338-X or the like. Examples of the vector to be used include pBlueScript II vector (manufactured by Stratagene), pUC18/19 vector (manufactured by TAKARA SHUZO Co.,Ltd.), TA Cloning vector (manufactured by Invitrogen) and the like.

A nucleotide sequence of the cloned gene may be confirmed by the Maxam Gilbert method (described in Maxam, A.M. & W.Gilbert, Proc. Natl. Acad. Sci. USA, 74, 560, 1977 etc.) or the Sanger method (described in Sanger, F.& A.R.Coulson, J.Mol.Biol., 94, 441, 1975, Sanger, F, & Nicklen and A.R.Coulson., Proc. Natl. Acad. Sci. USA, 74, 5463, 1977 etc.). For the procedures, commercially available kits such as Termo Seqenase II dye terminator cycle sequencing kit (manufactured by Amersham Pharmacia), Dye Terminator Cycle Sequencing FS Ready Reaction Kit (manufactured by PE Biosystems Japan) and the like can be used.

(3) Construction of expression vector

An expression vector of a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase may be constructed by a conventional method (for example, method described in J. Sambrook, E., F., Frisch, T., Maniatis, Molecular Cloning 2nd edition, published by Cold Spring Harbor Laboratory Press etc.).

For example, A polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase may be incorporated into a vector which can be utilized in a host cell to be transformed, for example, a vector which contains genetic information required to be replicable in a host cell, can replicates autonomously, can be isolated and purified from a host cell, and has a detectable marker (hereinafter referred to as basic vector in some cases). As the basic vector, specifically, when a bacterium such as Escherichia coli is used as a host cell, for the example, a plasmid pUC119 (manufacture d by TAKARA SHUZO Co., Ltd.), phagemid pBluescriptII (manufactured by Stratagene) and the like may be used. When yeast is used as a host cell, for example, plasmids pACT2 (manufactured by Clontech), p415 CYC (ATCC87382), p415 ADH (ATCC87374) and the like may be used. When a plant cell is used as a host cell, for the example, a plasmid pBI221 (Clontech) and the like may be used.

An expression vector which can express a polynucleotide

having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase in a host cell can be constructed by incorporating into a basic vector a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase upstream of which a promoter functional in a host cell is operably linked. Herein, the "operably linked" means that the promoter and a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kanase are ligated so that the polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase is expressed under control of the promoter in a host cell. Examples of a promoter functional in a host cell include, when a host cell is Escherichia coli, a promoter of a lactose operon (lacP) a promoter of tryptphan operon (trpP), a promoter of an arginine operon (argP), a promoter of a galactose operon (galP), tac promoter, T7 promoter, T3 promoter of Escherichia coli, a promoter of λ phage (λ-pL, λ-pR) and the like. In addition, when a host cell is yeast, examples include an ADH1 promoter, a CYC1 promoter and the like. The ADH1 promoter can be prepared, for example, by the conventional genetic engineering method from a yeast expression vector p415 ADH (ATCC87374) harboring an ADH1 promoter and a CYC1 terminator. The CYC1 promoter can be prepared by the conventional genetic engineering method from p415CYC (ATCC87382). Examples of the promoter include, when a host cell is a plant cell, a nopaline synthase gene (NOS) promoter, an

octopine synthase gene (OCT) promoter, a cauliflower mosaic virus (CaMV)-derived 19S promoter, a CaMV-derived 35S promoter and the like.

In addition, when a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase is incorporated into a vector already harboring a promoter functional in a host cell, a gene of the present histidine kinase may be inserted into downstream of the promoter so that a promoter harbored by the vector and a gene of the present histidine kinase are operably linked. For the example, the aforementioned yeast plasmid p415 ADH has an ADH1 promoter and, when a gene of the present histidine kinase is inserted downstream of an ADH1 promoter of the plasmid, an expression vector which can express a gene of the present histidine kinase in a budding yeast such as Saccharomyces cerevisiae AH22 (IFO10144) and TM182 (Maeda, T. et al. (1994) Nature 369:242-245) can be constructed.

(4) Preparation of transformed cell

By introducing the constructed expression vector into a host cell according to the conventional method, a transformed cell expressing the present histidine kinase can be prepared. As a host cell used for preparing such the transformed cell, for example, there are bacterium, yeast, plant cell and the like. As the bacterium, for example, there are Escherichia coli, Vibrio harveiy and the like. As the yeast, there are budding yeast and diving yeast. More specifically, for example, there are

yeasts belonging to genus Saccharomyces, genus Shizosaccharomycess the like. As a plant cell, for example, there is a plant cell such as Arabidopsis thaliana and the like.

As a method of introducing an expression vector into the aforementioned host cell, the conventional introducing method can be applied depending on a host cell to be transformed. For example, when bacterium is used as a host cell, the expression vector can be introduced into a host cell by the conventional introducing method such as a calcium chloride method and an electroporation method described in Moleculer Cloning (J. Sambrook et al., Cold spring Harbor, 1989). When yeast is used as a host cell, for example, the expression vector can be introduced into a host cell using Yeast transformation kit (Clontech) based on a lithium method. In addition, when a plant cell is used as a host cell, for example, the expression vector can be introduced into a host cell using the conventional introducing method such as an Agrobacterium infection method (JP-B No.2-58917 and JP-A No.60-70080), an electroporation method into a propoplast (JP-A No. 60-251887 and JP-A No. 5-68575) and a particle gun method (JP-A No.5-508316 and JP-A No.63-258525).

(Intracellular signal transduction system regarding present histidine kinase)

In the present invention, in order to measure an amount

of intracellular signal transduction from the present histidine kinase expressed in the transformed cell prepared as described above or an index value having the correlation therewith, an intracellular signal transduction system originally contained in a host cell used for preparing the transformed cell may be utilized. Examples of the intracellular signal transduction system which can be utilized include an intracellular signal transduction system regarding osmolarity responses of the aforementioned budding yeast, an intracellular signal transduction system regarding cell cycle progression and oxidative stress response of fission yeast, an intracellular signal transduction system regarding control of expression of capsular polysaccharide biosynthesis operon in Escherichia coli, an intracellular signal transduction system regarding control of cell density-sensitive luminescence of bioluminescent marine microorganism Vibrio harveyi, an intracellular signal transduction system regarding cytokinin response of Arabidopsis thaliana and the like.

When the aforementioned expression vector of the present histidine kinase is introduced using the "cell deficient in at least one hybrid-sensor kinase" as a host cell used for preparing such the transformed cell, the produced present histidine kinase functions in place of deleted hybrid-sensor kinase, and intracellular signal is transmitted. In the case where a test substance is contacted with the transformed cell, when signal

transduction from the present histidine kinase is inhibited by the test substance, change in an amount of growth of the transformed cell, change in morphology of the transformed cell, change in a shape of the transformed cell, change in an amount of biosynthesis of a particular substance in the cell, change in an amount of metabolism of a particular substance in the cell and the like occur in some cases. In such the cases, an antifungal activity of the test substance acting on the present histidine kinase can be measured using change in an amount of growth of the transformed cell, change in morphology, change in shape, change in an amount of biosynthesis of a particular substance in a cell, change in an amount of metabolism of a particular substance and the like as an index.

On the other hand, when at least one intrinsic hybrid-sensor kinase is not deleted in a host cell used for preparing a transformed cell, there are both of signal transduction from intrinsic hybrid-sensor kinases and intracellular signal transduction from the introduced present histidine kinase in intracellular signal transduction of the transformed cell. Change in an amount of growth of the transformed cell, change in morphology, change in shape, change in am amount of biosynthesis of a particular substance in the cell, change in an amount in metabolism of a particular substance in the cell and the like reflecting an amount of intracellular signal transduction from the introduced present histidine kinase

become smaller by the influence of an amount of intracellular signal transduction from intrinsic hybrid-sensor kinase. In the present invention, by using a host cell deficient in at least one intrinsic hybrid-sensor kinase, since change in an amount of growth of the transformed cell, change in morphology, change in shape, change in an amount of biosynthesis of a particular substance in the cell, change in an amount of metabolism of particular substance in the cell and the like reflecting an amount of intracellular signal transduction from the introduced present histidine kinase become larger, the sensitivity of the transformed cell to an antifungal compound is enhanced. Like this, the transformed cell with the enhanced sensitivity to an antifungal compound is useful for assaying the antifungal activity of a test substance and searching an antifungal compound using the assay.

Specifically, when the present histidine kinase is introduced in a Saccharomyces cerevisiae strain deficient in hybrid-sensor kinase SLN1 (Maeda, T. et al. Nature:369 242-245 (1994)), the present histidine kinase performs signal transduction in place of deficient SLN1, whereby, an amount of intracellular signal transduction from the introduced present histidine kinase can be detected more clearly using an amount of growth of host cell as an index. That is, when the test substance acts on the present histidine kinase, and an amount of signal transduction from the present histidine kinase in a

host cell is changed, it can be clearly measured as change in an amount of growth of the transformed budding yeast. In addition, an Escherichia coli strain deficient in a hybrid-sensor kinase RcsC, a fission yeast strain deficient in PHK1 to PHK3 involved in control of cell cycle progression, a Vibrio harveyi strain deficient in LuxN associated with control of cell density-sensitive luminescence and an Arabidopsis thaliana strain deficient in cytokinin receptor CRE1 can be exemplified as one preferable aspect of the "cell deficient in at least one hybrid-sensor kinase".

(Method of assaying antifungal activity of test substance)

In a method of assaying the antifungal activity of a test substance, an embodiment of a first step of culturing a transformed cell in which a polynucleotide having a nucleotide sequence encoding an amino acid sequence of the present histidine kinase introduced in the presence of a test substance includes a method of contacting a test substance with the transformed cell by culturing the transformed cell in a medium containing the test substance. Culturing the transformed cell may be any form of liquid culturing in which the cell is cultured in a liquid medium, solid culturing in which the cell is cultured on a solid medium prepared by adding agar or the like to liquid medium, and the like. The concentration of a test substance in the medium is, for example, about 1 nm to about 1 mM, preferably about 10

nm to about 100 μM. A culturing time is, for example, about 1 hour or longer and around 3 days, preferably about 25 hours to around 2 days. When the antifungal activity of a test substance is assayed, as a medium containing a test substance, an antifungal compound-free medium may be used.

An amount of intracellular signal transduction from the present histidine kinase expressed in a transformed cell cultured in the first step or an index value having the correlation therewith is measured. And, the antifungal activity of a test substance is assayed based on a difference between an amount of intracellular signal transduction or an index value having the correlation therewith measured in the second step and a control. For example, the antifungal activity of the test substance can be assessed based on a difference obtained by comparing amounts of intracellular signal transduction or index values having the correlation therewith, which are measured as described above in sections in which different two or more substances (for example, it is preferable that among different two or more substances, at least one substance has no antifungal activity) are independently used, respectively, as a test substance.

Specifically, for example, when a transformed cell prepared by using, as a host cell, the TM182 (SLN1Δ) strain (Maeda T. et al. Nature:369 242-245(1994)) which is a SLN1 gene-deficient strain in which the PTP2 Tyrosine phosphatase

gene (Ota et al, Proc.N.A.sic.USA, 89, 2355-2359(1992)) introduced (that is, a transformed cell having the function that cell growth is directly controlled by transduction of an intracellular signal from the present histidine kinase) is used, the antifungal activity can be measured by using, as an index, an amount of growth of the transformed cell in a medium (agar medium or liquid medium) using glucose as a carbon source, for example, Glu-Ura-Leu medium. When a medium in which a test substance is added to the Glu-Ura-Leu medium (medium containing no antifungal compound) is used, a test substance inhibiting growth of the transformed cell can be assessed to have the antifungal activity. In addition, as a control, it is enough to examine that growth of the transformed cell in a medium using galactose in place of glucose as a carbon source, for example, Gal-Ura-Leu medium is observed regardless of the presence or the absence of test substance.

When a transformed cell prepared by using, as a host cell, fission yeast which is PHK1, PHK2 and PHK3 gene-deficient strain (that is, a transformed cell in which cell cycle progression is directly regulated by transduction of an intracellular signal from the histidine kinase) is used, cell diviision of the fission yeast may be observed under a microscope. When a medium in which a test substance is added to a medium containing no substance having the antifungal activity is used, a test substance which shortens a cell length of a dividing cell of the transformed

cell can be assessed to have the antifungal activity.

When a transformed cell prepared by using, as a host cell, RcsC gene-deficient Escherichia coli in which cps-LacZ introduced is used, color development of X-Gal may be observed in an agar medium or a liquid medium (Suzuki et al. Plant Cell Physiol. 42:107-113(2001)). When a medium in which a test substance is added to a medium containing no substance having the antifungal activity is used, a test substance which can make the transformed cell develop blue can be assessed to have the antifungal activity.

In addition, when a transformed cell prepared by using, as a host cell, LuxN gene-deficient V. harveyi (i.e. a transformed cell in which bioluminescence is directly regulated by transduction of an intracellular signal from the present histidine kinase) is used, the fluorescent light emitted by the transformed microorganism may be observed. When a medium containing a test substance and not containing a substance having the antifungal activity is used, a test substance which make the transformed cell possible to emit the fluorescent light can be assessed to have the antifungal activity.

Further, a substance having the antifungal activity can be also searched by selecting an antifungal compound based on the antifungal activity assessed by the aforementioned assaying method.

Effects of the invention

The present invention can provide a transformed cell with the enhanced sensitivity to an antifungal compound, a method of assaying the antifungal activity of a test substance using the transformed cell, and a method of searching an antifungal compound using the method.

Examples

The present invention is further described in the following Examples, which are not intended to restrict the invention.

Example 1

Isolation of Botryotinia fuckeliana BcOS-1 gene

Total RNA was prepared from Botryotinia fuckeliana. 100 mg of a hypha of Botryotinia fuckeliana strain Bc-16 grown on a potato dextrose agar medium (PDA medium manufactured by NISSUI Pharmaceutical Co., Ltd.) was scratched off, and this was ground in liquid nitrogen using a mortar and a pestle. A RNA was prepared from frozen ground powder using RNeasy Plant Mini Kit (QIAGEN). A frozen ground powder together with liquid nitrogen was transferred to a 50 ml sample tube and, after liquid nitrogen was all volatilized, a solution obtained by adding 10 μL of mercaptoethanol per 1 ml of a buffer RLC attached to kit was added, followed by stirring. Further, ground powder was well dispersed by a few of pipettings, and was incubated at 56°C for

3 minutes. Thereafter, the solution containing ground powder was supplied to QIAshredder spin column attached to the kit, and centrifuged at 8,000 ×g for 2 minutes. The filtration supernatant was transferred to a fresh sample tube, a 0.5-fold volume of 99.5% ethanol was added thereto, and the material was well mixed by pipetting. This mixture was supplied to RNeasy mini spin column attached to the kit, and centrifuged at 8,000×g for 1 minute. The filtrate was discarded, the residue was added 700 μL of a buffer RW1 attached to the kit, and centrifuged at 8,000×g for 1 minute, and the filtrate was discarded. Further, the residue was added 500 μL of a buffer RPE attached to the kit, centrifuged at 8,000×g for 1 minute, and the filtrate was discarded. This procedure was repeated twice. Finally, an upper filter part was transferred to a fresh sample tube, supplied 30 μL of RNase-free sterilized water attached to the kit, and centrifuged at 8,000×g for 1 minute, and total RNA was dissolved out into the filtrate. This dissolution procedure was repeated twice. The concentration of the resulting total RNA solution was obtained from the absorbance at 260 nm to be 322 μg/ml.

Then, a cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen) while employing total RNA as a template. A solution in which 2.7 μL of total RNA and 6.3 μL of sterilized distilled water were mixed into 1.0 μL of 50 mM Oligo $(dt)_{20}$ attached to the kit and 2.0 μL of 10 mM dNTP Mix was treated at 65°C for 5 minute, and then rapidly cooled on ice. To this solution were

added 4 μL of 5×cDNA Synthesis Buffer attached to the kit, 1 μL of 0.1M DTT, 1 μL of RNase OUT, 1 μL of ThermoScript RT and 1 μL of sterilized distilled water, to react them at 50°C for 60 minutes and, thereafter, the reaction was stopped by heating treatment at 85°C for 5 minutes. Further, a RNA of a template was degraded by adding 1 μL of RNaseH attached to the kit to this reaction solution and maintained a temperature at 37°C for 20 minutes, to obtain a cDNA.

A DNA having a nucleotide sequence encoding an amino acid sequence of Botryotinia fuckiliana BcOS-1 (hereinafter, referred to as BcOS-1 DNA in some cases) was amplified by PCR using this cDNA as a template. Using an oligonucleotide comprising the nucleotide sequence represented by SEQ ID NO: 3 and an oligonucelotide consisting of the nucleotide sequence represented by SEQ ID NO: 4 as a primer, a PCR was performed to amplify a DNA having the nucleotide sequence represented by SEQ ID NO: 2. The PCR was performed using KOD-Plus-(TOYOBO) under the amplifying conditions that a temperature was maintained at 94°C for 2 minutes and, thereafter, 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds, then, at 68°C for 6 minutes. The PCR reaction solution (50 μL) was prepared by adding 2 μL of the aforementioned cDNA, 5 μL of 10×Buffer, 5 μL of 2 mM dNTPs, 2 μL of 25 mM $MgSO_4$, each 1 μL of 10 μM oligonucleotide primers, 33 μL of sterilized distilled water

and 1 μL of KOD-Plus-. After the reaction, a part of the reaction solution was separated by 0.8% agarose gel electrophoresis, and stained with ethidium bromide. It was confirmed that about 4 kb of a DNA (BcOS-1 DNA) was amplified.

Example 2

Construction of expression plasmid of Botryotinia fuckeliana BcOS-1 gene and preparation of transformed budding yeast

BcOS-1 DNA was cloned into a shuttle vector p415ADH (ATCC87312) replicable in yeast and Escherichia coli. About 4 kb of the aforementioned DNA (BcOS-1 DNA) was purified from the PCR reaction solution prepared in Example 1 using QIAquick PCR Purification Kit (QIAGEN) according to the attached manual. About 4 kb of the purified DNA (BcOS-1 DNA) was digested with restriction enzymes SpeI and PstI and, on the other hand, the shuttle vector p415ADH was also digested with restriction enzymes SpeI and PstI and, thereafter, each of which was separated by 0.8% agarose gel electrophoresis, and a part of the gel containing a desired DNA was excised. The BcOS-1 DNA digested with SpeI and PstI and the shuttle vector digested with SpeI and PstI were recovered from the gel using QIAquick Gel Extraction Kit (QIAGEN) according to the attached manual. The aforementioned BcOS-1 DNA was inserted between SpeI site and PstI site in the multicloning site of the shuttle vector using Ligation Kit Ver.2 (TaKaRa) according to the attached manual, to construct an

expression plasmid pADHBcOS1. A nucleotide sequence of the resulting expression plasmid was analyzed with a DNA sequencer (Model 3100, Applied Biosystems) after a sequencing reaction using BigDye terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the attached manual. The sequencing reaction was performed using an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NOs: 5 to 12 as a primer under the amplifying conditions that 30 cycles were repeated, each cycle comprising maintaining a temperature at 96°C for 10 seconds, then, at 50°C for 5 seconds, then, at 60°C for 4 minutes. As a result, the nucleotide sequence represented by SEQ ID NO: 2 was obtained, and it was confirmed that the expression plasmid pADHBcOS1 harbored a DNA having a nucleotide sequence encoding an amino acid sequence of BcOS-1.

The prepared expression plasmid pADHBcOS1 was introduced into each of budding yeast (Saccharomyces cerevisiae) AH22 strain (IFO10144) and TM182 strain (Maeda T. et al. (1994) Nature vol.369, pp242-245) according to the method described in Geitz RD & Woods RA (1994) Molecular Genetics of Yeast: Practical Approaches ed. Johnson JA, Oxford University Press pp124-134. By utilizing disappearance of leucine auxotrophy in the resulting transformed budding yeast, the transformed budding yeast AH 22 strain (AH22-BcOS1) was selected on a Glu-Leu agar medium, and the transformed budding yeast TM182 strain (TM182-BcOS1) was selected on a Gal-Ura-Leu agar medium. It was confirmed that

the resulting TM182-BcOS1 grows even when transplanted to a Glu-Ura-Leu medium.

Example 3

Antifungal compound sensitivity test of transformed budding yeast TM182-BcOS1

The transformed budding yeast AH22-BcOS1 prepared in Example 2 was cultured while shaking at 30°C for 18 hours in a Glu-Leu medium. As a control, the AH22 strain was similarly cultured while shaking at 30°C for 18 hours in a Glu medium. The absorbance at 600 nm of each of the grown transformed budding yeasts in a cell suspension was measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast AH22-BcOS1 was diluted 200-fold with a Glu-Leu medium, and a cell suspension in which the aforementioned cell suspension of the AH22 strain was diluted 200-fold with a Glu medium were prepared. A solution in which each of Compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 60 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 2000 ppm, and a solution in which each of Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in

which each 2.0 μL per well of the Compound solution and DMSO as a control were dispensed into two wells. In one microplate among them, each 200 μL of cell suspensions of the transformed budding yeast AH22-BcOS1 which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30°C for 48 hours. In another microplate, each 200 μL of the cell suspensions of the control yeast AH22 strain which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30°C for 48 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

Similarly, the transformed budding yeast TM182-BcOS1 prepared in Example 2 was cultured at 30°C for 18 hours in a Glu-Ura-Leu medium. The absorbance at 600 nm of the grown transformed budding yeast in a cell suspension was measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast TM182-BcOS1 was diluted 200-fold with a Glu-Ura-Leu medium and, as a control, a cell suspension in which the aforementioned cell suspension was diluted 200-fold with a Gal-Ura-Leu medium were prepared. A suspension in which each of Compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 60 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO)

to the concentration of 2000 ppm, and a solution in which each of Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 2.0 μL per well of the Compound solution and DMSO as a control were dispensed into two wells. In one microplate among them, each 200 μL of cell suspensions of the transformed budding yeast TM182-BcOS1 which had been prepared by dilution with a Glu-Ura-Leu medium as described above was dispensed, and cultured by allowing to stand at 30°C for 67 hours. In another microplate, as described above, as a control, each 200 μL of the cell suspensions of the transformed budding yeast TM182-BcOS1 which had been prepared by dilution with a Gal-Ura-Leu medium was dispensed, and cultured by allowing to stand at 30°C for 67 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

Degree of growths of both of the transformed budding yeasts cultured under the presence of each of Compound (1) to (7) and budding yeast as a control therefor are shown in Table 1. Degree of growths of both of the transformed budding yeasts and budding yeasts as a control therefor are expressed by a relative value in percentage, letting the absorbance at 600 nm in a well having the concentration of the aforementioned Compound of 0 ppm to be 100. It was confirmed that an inhibiting degree of growth of TM182-BcOS1 by each test substance was grater than an inhibiting degree of growth of AH22-BcOS1 by each test substance,

and the TM182-BcOS1 was a transformed cell with the enhanced sensitivity to an antifungal compound as compared with AH22-BcOS1.

Table 1

	Degree of growth of budding yeast (%)			
	AH22	AH22-BcOS1	TM182-BcOS1	
Test substance (final concentration)	Glu medium	Glu-Leu medium	Gal-Ura-Leu medium	Gal-Ura-Leu medium
Compound (1) (0.6 ppm)	99	90	99	9
Compound (2) (0.6 ppm)	99	92	98	11
Compound (3) (0.6 ppm)	98	93	98	10
Compound (4) (20 ppm)	96	45	102	10
Compound (5) (20 ppm)	97	79	103	48
Compound (6) (0.2 ppm)	99	81	99	8
Compound (7) (0.2 ppm)	101	94	99	11

Example 4

Isolation of Botryotinia fuckeliana mutant BcOS-1 gene exhibiting resistance to dicarboxyimide antifungal compound

A DNA having a nucleotide sequence encoding an amino acid sequence of Botryotinia fuckeliana mutant BcOS-1 (Oshima, M. et al. (2002) Phytopathology 92, pp75-80) exhibiting resistance to a dicarboxyimide antifungal compound (hereinafter, referred to as mutant BoOS1 DNA in some cases) was prepared by PCR using the cDNA prepared in Example 1 as a template. A first time PCR was performed using, as a primer, an oligonucelotide consisting of the nucleotide sequence represented by SEQ ID NO:

15 and an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 4, and a DNA having a nucleotide sequence represented by base numbers 1081 to 3948 of the nucleotide sequence represented by SEQ ID NO: 14 was amplified. The PCR was performed using KOD-Plus-(TOYOBO) under the amplifying conditions that a temperature was maintained at 94°C for 2 minutes and, thereafter, 35 cycles were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds, then, at 68°C for 6 minutes. The PCR reaction solution (50 μL) was prepared by adding 2 μL of the aforementioned cDNA, 5 μL of 10×Buffer, 5 μL of 2 mM dNTPs, 2 μL of 25 mM $MgSO_4$, each 1 μL of 10 μM oligonucleotide primers, 33 μL of sterilized distilled water and 1 μL of KOD-Plus-. After the reaction, a second PCR was performed using an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 3 and 1 μL of the first time PCR reaction solution while using the cDNA prepared in Example 1 as a template. The reaction conditions were the same as those of the first time PCR and after the reaction, a part of the reaction solution was separated by 0.8% agarose gel electrophoresis, and stained with ethidium bromide. It was confirmed that about 4 kb of the DNA (mutant BcOS-1 DNA) was amplified.

Example 5

Construction of expression plasmid of Botryotinia fuckeliana

BcOS-1 mutant gene exhibiting resistance to dicarboxyimde antifungal compound and preparation of transformed budding yeast

First, the mutant BcOS-1 DNA was cloned into a vector pBluescript II SK(+) (TOYOBO). About 4 kb of the DNA (mutant BcOS-DNA) was purified from the second time PCR reaction solution prepared in Example 4 using QIAquick PCR Purification Kit (QIAGEN) according to the attached manual. About 4 kb of the purified DNA (mutant BcOS-1 DNA) was digested with restriction enzymes SpeI and PstI and, on the other hand, the vector pBluescript II SK(+) was also digested with restriction enzymes SpeI and PstI, each of which was separated by 0.8% agarose gel electrophoresis, and a part of the gel containing a desired DNA was excised. The mutant BcOS-1 DNA digested with SpeI and PstI and the vector pBluescript II SK(+) digested with SpeI and PstI were recovered from the gel using QIAquick Gel Extraction Kit (QIAGEN) according to the attached manual. The aforementioned mutant BcOS-1 DNA was inserted between SpeI site and PstI site in the multicloning site of the vector pBluescript II SK(+) using Ligation Kit Ver.2 (TaKaRa) according to the attached manual, to construct a plasmid pBcOS1-I 365S. A nucleotide sequence of the resulting plasmid was analyzed with a DNA sequencer (Model 3100, Applied Biosystems) after a sequencing reaction using BigDye terminator v3.0 Cycle Sequence FS Ready Reaction Kit (Applied Biosystems) according to the attached manual. The

sequencing reaction was performed by using an oligonucleotide consisting of the nucleotide sequences represented by any of SEQ ID NOs: 7 to 12 as a primer under the amplifying conditions that 30 cycles were repeated, each cycle comprising maintaining a temperature at 96°C for 10 seconds, then, at 50°C for 5 seconds, then, at 60°C for 4 minutes. As a result, the nucleotide sequence represented by SEQ ID NO: 14 was obtained and it was confirmed that the plasmid pBcOS1-I 365S harbored the mutant BcOS-1 DNA.

The mutant BcOS-1 DNA contained in the thus prepared plasmid pBcOS1-I365S was cloned into a shuttle vector p415ADH replicable in yeast and Escherichia coli, to construct an expression plasmid. The plasmid pBcOS1-I365S was digested with restriction enzymes SpeI and PstI and, on the other hand, the shuttle vector p415ADH was also digested with restriction enzymes SpeI and PstI. These were separated by 0.8% agarose gel electrophoresis, respectively, each of gel parts containing the mutant BcOS-1 DNA digested with SpeI and PstI and the shuttle vector p415ADH digested with SpeI and PstI was excised, and the mutant BcOS-1 DNA and the shuttle vector were recovered from the gel using QIAquickGel Extraction Kit (QIAGEN) according to the attached manual. The mutant BcOS-1 DNA was inserted between SpeI site and PstI site in the multicloning site of the shuttle vector using Ligation Kit Ver.2 (TaKaRa) according to the attached manual, to construct an expression plasmid pADHBcOS1-I365S. A nucleotide sequence of the resulting

expression plasmid was analyzed with a DNA sequencer (Model 3100, Applied Biosystems) after a sequencing reaction using BigDye terminator v3.0 Cycle Sequence FS Ready Reaction Kit (Applied Biosystems) according to the attached manual. The sequencing reaction was performed by using an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NOs: 5 to 12 as a primer under the amplifying conditions that 30 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 96°C for 10 seconds, then, at 50°C for 5 seconds, then, at 60°C for 4 minutes. As a result, the nucleotide sequence represented by SEQ ID NO: 14 was obtained, and it was confirmed that the expression plasmid pADHBcOS1-I365S harbored a DNA having a nucleotide sequence encoding an amino acid sequence of the mutant BcOS-1.

The prepared expression plasmid pADHBcOS1-I 365S was introduced into the budding yeast TM182 strain according to the method described in Example 2. By utilizing disappearance of leucine auxotrophy in the resulting transformed budding yeast, the transformed budding yeast TM182 strain (TM182-BcOS1-I365s) was selected on a Gal-Ura-Leu agarose medium. It was confirmed that the resulting TM182-BcOS1-I365S grows even when transplanted to a Glu-Ura-Leu medium.

Example 6

Antifungal compound sensitivity test of transformed budding

yeast TM182-BcOS1-I-365S

The transformed budding yeast TM182-BcOS1-I365S prepared in Example 5 was cultured at 30°C for 18 hours in a Glu-Ura-Leu medium. The absorbance at 600 nm of a cell suspension of the grown transformed budding yeast was measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast TM182-BcOS1-I 365S was diluted 200-fold with a Glu-Ura-Leu medium and, as a control, a cell suspension in which the cell suspension was diluted 200-fold with a Gal-Ura-Leu medium were prepared. A solution in which each of Compound (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 60 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 2000 ppm, and a solution in which each of Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 2.0 μL per well of the Compound solution and DMSO as a control were dispensed into two wells. In one microplate among them, each 200 μL of cell suspensions of the transformed budding yeast TM182-BcOS1-I365S which had been prepared by dilution with a Glu-Ura-Leu medium as described above was dispensed, and cultured by allowing to stand at 30°C for 67 hours. In another microplate, as a control, each 200 μL of

cell suspensions of the transformed budding yeast TM182-BcOS1-I 365S which had been prepared by dilution with a Gal-Ura-Leu medium was dispensed, and cultured by allowing to stand at 30°C for 67 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

Degrees of growths of both of the transformed budding yeasts cultured under the presence of Compounds (1) to (7) and budding yeast as a control therefor are shown in Table 2. Degrees of growths of both of the transformed budding yeasts and budding yeast as a control are expressed by a relative value in percentage, letting the absorbance at 600 nm at the concentration of the Compound of 0 ppm to be 100. It was confirmed that an inhibiting degree of growth of the transformed budding yeast TM182-BcOS1-I 365S by each test substance was grater than an inhibiting degree of growth of the transformed budding yeast AH22-BcOS1-I 365S by each test substance, and the transformed budding yeast TM182-BcOS1-I 365S was a transformed cell with the enhanced sensitivity to an antifungal compound as compared with the transformed budding yeast AH22-BcOS1-I365S.

Table 2

	Degree of growth of budding yeast (%)			
	AH22	AH22-BcOS1-I365S	TM182-BcOS1-I365S	
Test substance (final concentration)	Glu medium	Glu-Leu medium	Gal-Ura-Leu medium	Glu-Ura-Leu medium
Compound (1) (6 ppm)	88	68	99	9
Compound (2) (6 ppm)	91	81	88	11
Compound (3) (6 ppm)	87	75	92	9
Compound (4) (20 ppm)	96	83	101	41
Compound (5) (20 ppm)	80	64	76	13
Compound (6) (0.2 ppm)	92	67	93	7
Compound (7) (0.2 ppm)	91	79	90	22

Example 7

Isolation of Magnaporthe grisea HIK1 gene

Total RNA was prepared from Magnaporthe grisea. 100 mg of a hypha of Magnaporthe grisea P-37 strain which had been grown on a potato dextrose agar medium (PDA medium manufactured by NISSUI Pharmaceutical Co., Ltd.) was scratched off, and this was ground using a mortar and a pestle in liquid nitrogen. A RNA was prepared from frozen ground powder using RNeasy Plant Mini Kit (QIAGEN). A frozen ground powder together with liquid nitrogen was transformed to a 50 ml sample tube and, after liquid nitrogen was all volatilized off, a solution obtained by adding 10 μL of mercaptoethanol was added per 1 ml of a buffer RLC attached to the kit was added, followed by stirring. Further, after ground

powder was well dispersed by a few pipettings, a temperature was maintained at 56°C for 3 minutes. Thereafter, a solution containing ground powder was supplied to QIAshredder spin column attached to the kit, and centrifuged at 8,000×g for 2 minutes. The filtration supernatant was transferred to a fresh sample tube, a 0.5-fold volume of 99.5% ethanol was added, and the material was well mixed by pipetting. This mixtured solution was supplied to RNeasy mini spin column attached to the kit, and centrifuged at 8,000×g for 1 minute. The filtrate was discarded, 700 μL of Buffer RW1 attached to the kit was added, centrifuged at 8,000×g for 1 minute, and the filtrate was discarded. Further, the residue was added 500 μL of Buffer RPE attached to the kit, and centrifuged at 8,000×g for 1 minute, and the filtrate was discarded. This procedure was repeated twice. Finally, an upper filter part was transferred to a fresh sample tube, supplied 30 μL of RNase-free sterilized water, and centrifuged at 8,000×g for 1 minute, and total RNA was dissolved into the filtrate. This dissolution procedure was repeated twice.

Then, a cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen) while using total RNA as a template. A solution in which 9.0 μL of total RNA was mixed into 1.0 μL of 50 mM Oligo$(dt)_{20}$ attached to the kit and 2.0 μL of 10 mM dNTP Mix was treated at 65°C for 5 minutes, and rapidly cooled on ice. To this solution were added 4 μL of 5×cDNA Synthesis Buffer

attached to the kit, 1 μL of 0.1M DTT, 1 μL of RNase OUT, 1 μL of ThermoScript RT and 1 μL of sterilized distilled water, to react them at 50°C for 60 minutes and, thereafter, the reaction was stopped by heating treatment at 85°C for 5 minutes. Further, 1 μL of RNaseH attached to the kit was added to this reaction solution, the materials were reacted at 37°C for 20 minutes, and a RNA as a template was degraded to obtain a cDNA.

A DNA having a nucleotide sequence encoding an amino acid sequence of Magnaporthe grisea HIK1 (hereinafter, referred to as HIK1 DNA in some cases) was amplified by PCR using this cDNA as a template. A PCR was performed using an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 18 and an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 19, to amplify a DNA having the nucleotide sequence represented by SEQ ID NO: 17. The PCR was performed using KOD-Plus-(TOYOBO) under the amplifying conditions that a temperature was maintained at 94°C for 2 minutes and, thereafter, 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds, then, at 68°C for 6 minutes. The PCR reaction solution (50 μL) was prepared by adding 2 μL of the aforementioned cDNA, 5 μL of 10×Buffer, 5 μL of 2 mM dDNPs, 2 μL of 25 mM $MgSO_4$, each 1 μL of 10 μM oligonucleotide primers, 33 μL of sterilized distilled water and 1 μL of KOD-Plus-. After the reaction, a part of the reaction solution was separated with 1.0% agarose

gel electrophoresis, and stained with ethidium bromide. It was confirmed that about 4 kb of the DNA (HIK1 DNA) was amplified.

Example 8

Construction of an expression plasmid of Magnaporthe grisea HIK1 gene and preparation of transformed budding yeast

The HIK1 DNA was cloned into a cloning vector pBluesripit SK II (+). About 4 kb of the aforementioned DNA (HIK1 DNA) was purified from the PCR reaction solution prepared in Example 7 using QIAquick PCR Purification Kit (QIAGEN) according to the attached manual. About 4 kb of the purified DNA (HIK1 DNA) was digested with restriction enzymes of SpeI and HindIII and, on the other hand, after the cloning vector pBluescript SK II (t) (manufactured by Stratagene) was also digested with restriction enzymes SpeI and HindIII, each of which was separated with 1.0% agarose gel electrophoresis, and a part of the gel containing a desired DNA was excised. The NIK1 DNA digested with SpeI and HindIII and the cloning vector digested with SpeI and HindIII were recovered form the gel using QIAquick Gel Extraction Kit (QIAGEN) according to the attached manual. The HIK1 DNA was inserted between SpeI site and HindIII site in the multicloning site of the cloning vector using Ligation Kit Ver.2 (TaKaRa) according to the attached manual, to construct a plasmid pBlueHIK1. A nucleotide sequence of the resulting plasmid was analyzed with a DNA sequencer (Model 3100, Applied Biosystems)

after a sequencing reaction using BigDye terminator v3.0 Cycle Sequence FS Ready Reaction Kit (Applied Biosystems) according to the attached manual. The sequencing reaction was performed using an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NOs:20 to 29 as a primer under the amplifying conditions that 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 96°C for 10 seconds, then, at 50°C for 5 seconds, then, at 60°C for 2 minutes. As a result, the nucleotide sequence represented by SEQ ID NO: 17 was obtained, and it was confirmed that the plasmid pBlueHIK1 harbored a DNA having a nucleotide sequence encoding an amino acid sequence of HIK1.

Then, the HIK1 DNA was inserted into a shuttle vector p415ADH (ATCC87312) replicable in yeast and Escherichia coli. The plasmid pBlueHIK1 prepared as described above was digested with restriction enzymes SpeI and HindIII and, on the other hand, after the shuttle vector p415ADH (ATCC87312) was also digested with restriction enzymes SpeI and HindIII, each of which was separated with 1.0% agarose gel electrophoresis, and a part of the gel containing a desired DNA was excised. The HIK1 DNA digested with SpeI and HindIII and the shuttle vector digested with SpeI and HindIII were recovered from the gel using QIAquick Gel Extraction Kit (QIAGEN) according to the attached manual. The HIK1 DNA was inserted between SpeI site and HindIII site in the multicloning site of the shuttle vector using Ligation

Kit Ver.2 (TaKaRa) according to the attached manual, to construct an expression plasmid pADHHIK1.

The prepared expressed plasmid pADHHIK1 was introduced into budding yeast (Saccharomyces cerevisiae) AH22 strain (IFO10144) and TM182 strain (Maeda T. et al. (1994) Nature vol.369, pp242-245) according to the method described in Geitz RD & Woods RA (1994) Molecular Genetics of Yeast: Practical Approaches ed. Johnson JA, Oxford University Press pp124-134. By utilizing disappearance of leucine auxotrophy in the resulting transformed budding yeast, the transformed budding yeast AH22 strain (AH21-HIK1) was selected on a Glu-Leu agar medium, and the transformed budding yeast TM182 strain (TM182-HIK1) was selected on a Glu-Ura-Leu agar medium. It was confirmed that the resulting TM182-HIK1 grows even transferred to a Glu-Ura-Leu medium.

Example 9

Antifungal compound sensitivity test of transformed budding yeast TM182-HIK1

The transformed budding yeast AH22-HIK1 prepared in Example 8 was cultured while shaking at 30°C for 24 hours in a Glu-Leu medium. As a control, the AH22 strain was similarly cultured while shaking at 30°C for 24 hours in a Glu medium. The absorbance at 600 nm of a cell suspension of each of the grown transformed budding yeasts was measured, and a cell suspension diluted with each medium to the absorbance of 0.1

was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast AH22-HIK1 was diluted 50-fold with a Glu-Leu medium, and a cell suspension in which the aforementioned cell suspension of AH22 strain was diluted 50-fold with a Glu medium were prepared. A suspension in which each of compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 200 ppm, a solution in which each of Compounds (4) to (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 600 ppm, and a solution in which each of Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 1.0 μL per well of the Compound solution and DMSO as a control were dispensed into two wells. In one microplate among them, each 100 μL of a cell suspension of the transformed budding yeast AH22-hiki which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30°C for 23 hours. In another microplate, each 100 μL of the cell suspensions of control yeast AH22 strain which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30°C for 27 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

Similarly, the transformed budding yeast TM182-HIK1 prepared in Example 8 was cultured at 30°C for 24 hours in a

Glu-Ura-Leu medium. The absorbance at 600 nm of a cell suspension of the grown transformed budding yeast was measured, and a cell suspension diluted with each medium to the absorbance of 0.1 was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast TM182-HIK1 was diluted 50-fold with a Glu-Ura-Leu medium and, as a control, a cell suspension in which the suspension was diluted 50-fold with a Glu-Ura-Leu medium were prepared. A suspension in which each of Compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 200 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 600 ppm, and a solution in which each of Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 1.0 μL per well of the Compound DMSO solution and DMSO as a control were dispensed into two wells. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast TM182-HIK1 which had been prepared by dilution with a Glu-Ura-Leu medium as described above was dispensed, and cultured by allowing to stand at 30°C for 27 hours. In another microplate, as described above, as a control, each 100 μL of cell suspensions of the transformed budding yeast TM182-HIK1 which had been prepared by dilution with a Gal-Ura-Leu medium was dispensed, and cultured by allowing to stand at 30°C for 27 hours. After culturing,

the absorbance at 600 nm of each well was measured with a microplate reader.

Degree of growths of both of the transformed budding yeasts cultured in the presence of Compounds (1) to (7) and budding yeast as a control therefor are shown in Table 3. Degrees of growths of both of the transformed budding yeasts and budding yeast as a control thereofor are shown by a relative value in percentage, letting the absorbance of 600 nm at the concentration of the Compound of 0 ppm to be 100. It was confirmed that an inhibiting degree of growth of TM182-HIK1 by each test substance was greater than an inhibiting degree of growth of AH22-HIK1 by each test substance, and the TM182-HIK1 was a transformed cell with the enhanced sensitivity to an antifungal compound as compared with AH22-HIK1.

Table 3

	Degree of growth of budding yeast (%)			
	AH22	AH22-HIK1	TM182-HIK1	
Test substance (final concentration)	Glu medium	Glu-Leu medium	Gal-Ura-Leu medium	Glu-Ura-Leu medium
Compound (1) (2.0 ppm)	85	89	100	62
Compound (2) (2.0 ppm)	96	84	94	79
Compound (3) (2.0 ppm)	99	104	100	30
Compound (4) (6.0 ppm)	97	92	97	63
Compound (5) (6.0 ppm)	93	99	106	22
Compound (6) (0.2 ppm)	101	98	104	11
Compound (7) (0.2 ppm)	89	102	87	9

Example 10

Amplification of osmosensitivie histidine kinase gene fragment from other filamentas fungus

(1) Preparation of Total RNA of Fusarium oxysporum

Total RNA was prepared from Fusarium oxysporum. 100 mg of a hypha of Fusarium oxyporum RJN1 strain grown on a potato dextrose agarose medium (PDA medium, manufactured by NISSUI Pharmaceutical Co., Ltd.) was collected, and this was ground using a mortar and a pestle in liquid nitrogen. Total RNA was prepared from frozen ground powder using RNeasy Plant Mini Kit (QIAGEN) according to the method described in Example 1.

(2) Preparation of Total RNA of Mycospharella tritici

Total RNA was prepared from Mycospharella tritici. Spore of Mycospharella tritici St-8 strain grown on a potato dextrose agarose medium (PDA medium, manufactured by NISSUI Pharmaceutical Co., Ltd.) was added to 100 ml of PD broth (DIFCO), and this was cultured at 20°C and 150rpm for 4 days using a 500 ml volume Erlenmeyer flask. 8 ml of the culture solution was centrifuged to remove the supernatant, and 300 mg of a wet weight of cells were transferred to a mortar and ground in liquid nitrogen using a pestle. Total RNA was prepared from frozen ground powder according to the method described in Example 1.

(3) Preparation of total RNA of Thanatephorus cucumeris

Total RNA was prepared from Thanatephorus cucumeris. Hypha of Thanatephorus cucumeris Rs-18 strain grown on a potato

dextrose agar medium (PDA medium, manufactured by NISSUI Pharmaceutical Co., Ltd.) was added to 100 ml of PD broth (DIFCO), and cultured by allowing to stand at 25°C for 4 days using a 500 ml volume Erlenmeyer flask. 8 ml of the culture solution was centrifuged to remove the supernatant, 300 mg of a wet weight of hypha were transferred to a mortar, and ground in liquid nitrogen using a pestle. Total RNA was prepared from frozen ground powder using Rneasy Plant Mini Kit (QIAGEN) according to the method described in Example 1.

(4) Preparation of total RNA of Phytophthora infestans

Total RNA was prepared from Phytophthora infestans. Hypha of Phytophthora infestans Pi-5 strain grown on a rye agar medium (rye 60g, sucrose 15g, agar 20g/1L) was added to 20 ml of a rye medium (rye 60g, sucrose 15g/1L), and cultured at 20°C and 150rpm for 5 days using a 300 ml of volume Erlenmeyer flask. 20 ml of the culture solution was centrifuged to remove the supernatant, a wet weight of 200 mg of cells were transferred to a mortar, and ground using a pestle in liquid nitrogen. Total RNA was prepared from frozen ground powder using RNeasy Plant Mini Kit (QIAGEN) according to the method described in Example 1.

(5) Amplification of osmosensing histidine kinase gene fragment by PCR

Using the total RNA of Magnaporthe grisea prepared in Example 7, the total RNA of Fusarium oxysporum prepared in Example

10 (1), the total RNA of Mycospharella tritici prepared in Example 10 (2), the total RNA of Thanatephorus cucumeris prepared in Example 10 (3), or the total RNA of Phytophthora infestans prepared in Example 10 (4), amplification of a DNA having a nucleotide sequence encoding a part of an amino acid sequence of osmosensing histidine kinase was performed.

First, a cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen) and using each of total RNAs as a template. A solution in which 4.0 μL of each of total RNAs and 5.0 μL of sterilized distilled water were mixed into 1.0 μL of 50 mM Oligo$(dT)_{20}$ attached to the kit and 2.0 μL of 10 mM dNTP Mix was prepared, and a cDNA was synthesized according to the method described in Example 1.

A PCR was performed using each cDNA as a template. As primers, a primer pair shown in Table 4 was used. A size of a DNA which is predicted to be amplified by PCR using each primer pair based on the nucleotide sequence represented by SEQ ID NO: 2 is shown in Table 4.

Table 4

Primer Pair	Primer	Primer	DNA to be amplified
1	SEQ ID NO: 30	SEQ ID NO: 35	368bp
2	SEQ ID NO: 30	SEQ ID NO: 36	374bp
3	SEQ ID NO: 30	SEQ ID NO: 37	383bp
4	SEQ ID NO: 31	SEQ ID NO: 35	359bp
5	SEQ ID NO: 31	SEQ ID NO: 36	365bp
6	SEQ ID NO: 31	SEQ ID NO: 37	374bp
7	SEQ ID NO: 32	SEQ ID NO: 38	3019bp
8	SEQ ID NO: 32	SEQ ID NO: 40	3052bp
9	SEQ ID NO: 33	SEQ ID NO: 38	2927bp
10	SEQ ID NO: 33	SEQ ID NO: 40	2960bp
11	SEQ ID NO: 34	SEQ ID NO: 38	2867bp
12	SEQ ID NO: 34	SEQ ID NO: 40	2900bp
13	SEQ ID NO: 30	SEQ ID NO: 39	1424bp
14	SEQ ID NO: 30	SEQ ID NO: 40	1442bp
15	SEQ ID NO: 31	SEQ ID NO: 39	1415bp
16	SEQ ID NO: 31	SEQ ID NO: 40	1433bp

A PCR was performed using KOD-Plus-(TOYOBO) under the amplifying conditions that a temperature was maintained at 94°C for 2 minutes and, thereafter, 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds further, at 68°C for 1 minutes. When primer pairs 1 to 6 were used, the incubation at 68°C in the cycle was for 1 minutes. When the primer pairs 7 to 12 were used, the incubation at 68°C in the cycle was for 5 minutes. When the primer pairs 13 to 16 were used, the incubation at 68°C in the cycle was for 3 minutes.

The PCR reaction solution (25 μL) was prepared by adding 0.5 μL of the cDNA, 2.5 μL of 10×buffer, 2.5 μL of 8 mM dNTPs, 1.0 μL of 25 mM $MgSO_4$, each 0.5 μL of 10 μM oligonucleotide primers, 17 μL of sterilized distilled water and 0.5 μL of KOD-Plus-. The PCR reaction solution after the reaction was analyzed with 1% or 4% agarose gel electrophoresis.

When primer pairs 1, 2, 3, 4, 5 or 6 were used and a cDNA of Magnaporthe grisea was used as a template, amplification of predicted size of DNA was observed. When primer pairs 2, 3,, 7, 8, 9, 10, 11 or 12 were used, and a cDNA of Fusarium oxysporum was used as a template, amplification of a predicted size of DNA was observed. When the primer pairs 3, 5, 6, 13, 14, 15 or 16 were used, and cDNA of Mycospharella Tritici was used as a template, amplification of predicted size of DNA was observed. When primer pairs 2, 3, 5 or 6 were used, and cDNA of Thanatephorus cucumeris was used as a template, amplification of a predicted size of a DNA was observed. When the primer pairs 5 or 6 were used, and cDNA of Phytophthora infestans was used as a template, amplification of predicted size of DNA was observed.

Example 11

Isolation of Fusarium oxysporum FoOS-1 gene

(1) Analysis of Fusarium oxysporum FoOS-1 gene fragment

The amplified DNA was purified from the reaction solution of PCR which had been performed by using a cDNA of Fusarium

oxysporum as a template and using a primer pair 9 in Example 10 (5), using QIAquick PCR Purification Kit (QIAGEN) according to the attached instruction.

Adenine was added to the 3'-terminal of the purified DNA using Ex Taq (TaKaRa) (hereinafter, referred to as 3'A addition). The reaction solution (20 μL) for 3' A addition was prepared by adding 15.3 μL of a solution of the aforementioned purified DNA, 2.0 μL of 10×buffer, 2.5 μL of 10 mM dNPTs and 0.2 μL of Ex Taq, and this was maintained at 72°C for 30 minutes.

Thus the 3'A-added DNA and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the instruction attached to the cloning vector, after that, which was introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA was purified from the resulted Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence of the plasmid DNA was analyzed with a DNA sequencer (Model 3100, Applied Biosystems) after a sequencing reaction employing the resulting plasmid DNA as a template, and using an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28, 29, and 45 to 48 as a primer, and using BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan) according to the instruction attached to the kit. The sequencing reaction was performed under the amplifying conditions that 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 96°C for 10 seconds,

then, at 50 °C for 5 seconds, further, at 60°C for 2 minutes. As a result, a nucleotide sequence represented by base numbers 663 to 3534 of the nucleotide sequence represented by SEQ ID NO: 42 was read.

(2) Analysis of full length FoOS-1 gene of Fusarium oxysporum

A DNA having a nucleotide sequence extending toward to the 5′ upstream region from a nucleotide number 663 of the nucleotide sequence represented by SEQ ID Mo. 42 was cloned using SMART RACE cDNA Amplification Kit (CLONTECH) according to the instruction attached to the kit. 1.0 μL of CDS-primer attached to the kit, and 1.0 μL of SMART IIA Oligo were mixed into 3 μL (230ng) of the total RNA prepared in Example 10 (1) to prepare a reaction solution. The reation solution was maintained at 70°C for 2 minutes and maintained on ice for 2 minutes. To the reaction solution were added 2 μL of 5 × First-Strand buffer attached to the kit, 1 μL of 20 mM DTT, 1 μL of 10 mM dNPT Mix and 1 μL of PowerScript Reverse Transcriptase and mixed, and the mixture was maintained at 42°C for 1.5 hours. To the reaction solution after temperature maintenance was added 100 μL of Tricine-EDTA buffer attached to the kit, and a temperature was maintained at 72°C for 7 minutes to prepare 5′ RACE ready cDNA. PCR amplifying 5′ upstream region was performed by using this 5′ RACE ready cDNA as a template. A PCR reaction solution was obtained by adding 5.0 μL of 10× Advantage 2 buffer, 1.0 μL of 10 mM dNTP Mix and 1.0 μL of 50× Advantage 2 Polymerase Mix attached

to the kit to 2.5 μL of 5' RACE ready cDNA and mixing them, and adding 5.0 μL of 10×Universal Primer A Mix attached to the kit as a primer, and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 43, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution was subjected to repetition of 5 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 72°C for 2 minutes, further repetition of 5 cycles of incubation, each cycle comprising a maintaining a temperature at 94°C for 5 seconds, then, at 70°C for 10 seconds, then, at 72°C for 2 minutes, further repetition of 25 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 68°C for 10 seconds, then, at 72°C for 2 minutes, followed by maintaining a temperature at 72°C for 7 minutes. The PCR reaction solution and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the instruction attached to the cloning vector, after that, which was introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA was purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence was analyzed using the resulting plasmid DNA as a template, and using a primer consisting of the nucleotide sequence represented by any of SEQ ID NOs: 29, 49 and 54 according to the method described in Example 11 (1). As a result, a nucleotide sequence represented by

nucleotide numbers 1 to 662 of the nucleotide sequence represented by SEQ ID NO: 42 was read.

Further, a DNA having a nucleotide sequence extending toward to the 3′ downstream region from nucleotide number 3534 of the nucleotide sequence represented by SEQ ID NO: 42 was cloned. 1.0 μL of CDS-primer attached to the kit and 1.0 μL of sterilized distilled water were mixed into 3 μL (230ng) of the total RNA prepared in Example 10 (1), the mixture was maintained at 70°C for 2 minutes, and maintained on ice for 2 minutes. 3′ RACE ready cDNA was prepared using the reaction solution as in preparation of 5′ RACE ready cDNA. PCR amplifying 3′ downstream region was performed using this 3′ RACE ready cDNA as a template. A PCR reaction solution was prepared by mixing 5.0 μL of 10× Advantage 2 buffer attached to the kit, 1.0 μL of 10 mM dNTP Mix and 1.0 μL of 50× Advantage 2 Polymerase Mix into 2.5 μL of 3′ RACE ready cDNA, adding 5.0 μL of 10×Universal Primer A Mix attached to the kit as a primer, and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 42, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution was subjected to repetition of 5 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 72°C for 2 minutes, further repetition of 5 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 70°C for 10 seconds, then, at 72°C for 2 seconds,

further repetition of 25 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 68°C for 10 seconds, then, at 72°C for 2 minutes, followed by maintaining a temperature at 72°C for 7 minutes. The PCR reaction solution and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated to the vector according to the instruction attached to the kit, after that, which was introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA was purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence was analyzed using the resulting plasmid DNA as a template, and using a primer consisting of the nucleotide sequence represented by any of SEQ ID NOs: 29, 50 and 54, according to the method described in Example 11 (1). As a result, a nucleotide sequence represented by nucleotide numbers 3535 to 3882 of the nucleotide sequence represented by SEQ ID NO: 42 was read.

All analyzed nucleotide sequences were joined and, as a result, the nucleotide sequence represented by SEQ ID NO: 42 was obtained. The nucleotide sequence represented by SEQ ID NO: 42 consists of 3882 bases (including termination codon), and was a nucleotide sequence encoding 1293 amino acid residues (SEQ ID NO: 41). A molecular weight of a protein having the amino acid sequence represented by SEQ ID NO: 41 was calculated to be 141818 Da.

(3) Isolation of full length Fusarium oxysporum FoOS1 gene

A DNA having a nucleotide sequence encoding an amino acid sequence of Fusarium oxysporum FoOS1 (hereinafter, referred to as FoOS-1 DNA in some cases) was amplified by PCR using the 5′ RACE ready cDNA prepared in Example 11 (2) as a template. By performing a PCR using, as a primer, an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 52 and an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 53, a DNA having the nucleotide sequence represented by SEQ ID NO: 42 was amplified. The PCR was performed using KOD-Plus- (TOYOBO) under the amplifying conditions that a temperature was maintained at 94°C for 2 minutes and, thereafter, 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 55°C for 30 seconds, further, at 68°C for 6 minutes. The PCR reaction solution (50 μL) was prepared by adding 2.5 μL of 5′ a RACE ready cDNA, 5.0 μL of 10×buffer, 5.0 μL of 2 mM dNTPs, 2.0 μL of 25 mM $MgSO_4$, each 1.0 μL of 10 μM oligonucleotide primers, 32.5 μL of sterilized distilled water and 1.0 μL of KOD Plus-. After the reaction, a part of the PCR reaction solution was separated by 1% agarose gel electrophoresis, and stained with ethidium bromide. It was confirmed that about 4 kb of the DNA (FoOS1 DNA) was amplified.

Example 12

Construction of expression plasmid of Fusarium oxysporum FoOS1

gene and preparation of transformed budding yeast

The FoOS1 DNA was cloned into a pCR2.1-TOPO cloning vector (Invitrogen). About 4 kb of the DNA (FoOS-1 DNA) was purified from the PCR reaction solution prepared in Example 11 (3) using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. 3'A addition was performed on about 4 kb of the purified DNA (FoOS-1 DNA) according to the method described in Example 11 (1). The 3'A-added about 4 kb DNA (FoOS-1 DNA) and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the manual attached to the cloning vector to construct a plasmid pCRFoOS1. A nucleotide sequence of the resulting plasmid was analyzed according to the method described in Example 11(1). As a primer, an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NOs: 29, 43 to 51, and 54 was used. As a result, the nucleotide sequence represented by SEQ ID NO: 42 was obtained, and it was confirmed that the plasmid pCRFoOS1 was a plasmid containing the FoOS-1 DNA.

The FoOS-1 DNA contained in the thus prepared plasmid pCRFoOS1 was cloned into a shuttle vector p415ADH replicable in yeastand Escherichia coli to construct an expression plasmid. The plasmid pCRFoOS1 was digested with restriction enzymes SpeI and PstI and, on the other hand, the shuttle vector p415ADH was also digested with restriction enzymes SpeI and PstI. Each of them was separated by 0.8% agarose gel electrophoresis, a part

of the gel containing the FoOS-1 DNA digested with SpeI and PstI and the shuttle vector p415ADH digested with SpeI and PstI was excised, and the FoOS-1 DNA and the shuttle vector were recovered from the gel using QIAquick Gel Extraction Kit (QIAGEN) according to the attached manual. The FoOS-1 DNA was inserted between SpeI site and PstI site in the multicloning site of the shuttle vector using Ligation Kit Ver.2 (TaKaRa) according to the manual attached to the kit, whereby, an expression plasmid pADHFoOS1 was constructed. A nucleotide sequence of the resulting expression plasmid was analyzed according to the method described in Example 11 (1). As a primer, an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NO: 43 to 53 was used. As a result, the nucleotide sequence represented by SEQ ID NO: 42 was obtained, and it was confirmed that the expression plasmid pADHFoOS1 harbored a DNA having a nucleotide sequence encoding an amino acid sequence of FoOS-1.

The prepared expression plasmid pADHFoOS1 was introduced into budding yeast AH22 strain and TM182 strain according to the method described in Example 2. By utilizing the disappearance of leucine auxotrophy in the resulting transformed budding yeast, the transformed budding yeast AH22 strain (AH22-FoOS1) was selected on a Glu-Leu agar medium, and the transformed budding yeast TM182 strain (TM182-FoOS1) was selected on a Gal-Ura-Leu agar medium. It was confirmed that the resulting TM182-FoOS1 grows even when transplanted to a

Glu-Ura-Leu medium.

Example 13

Antifungal compound sensitivity test of transformed budding yeast TM182-FoOS1

The transformed budding yeast AH22-FoOS1 prepared in Example 12 was cultured while shaking at 30°C for 18 hours in a Glu-Leu medium. As a control, the AH22 strain was similarly cultured while shaking at 30°C for 18 hours in a Glu medium. The absorbance at 600 nm of each grown transformed budding yeast in a cell suspension was measured, and cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast AH22-FoOS1 was diluted 50-fold with a Glu-Leu medium, and a cell suspension in which the aforementioned cell suspension of the AH22 strain was diluted 50-fold with a Glu medium were prepared.

A solution in which each of Compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 600 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 2000 ppm, and a solution in which Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 1.0 μL per well of the Compound solution and DMSO as a control

were dispensed. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast AH22-FoOS1 which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30 °C for 26.5 hours. In another microplate, each 100 μL of cell suspensions of the control yeast AH22 strain which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30°C for 24.5 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

Similarly, the transformed budding yeast TM182-FoOS1 prepared in Example 12 was cultured at 30°C for 18 hours in a Glu-Ura-Leu medium. The absorbance at 600 nm of the grown transformed budding yeast in a cell suspension was measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the transformed budding yeast TM182-FoOS1 was diluted 50-fold with a Glu-Ura-Leu medium and, as a control, a cell suspension in which the yeast was diluted 50-fold with a Gal-Ura-Leu medium were prepared.

A solution in which each of Compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 600 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 2000 ppm, and a solution in which each of Compounds (6) and (7)

was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 2.0 μL per well of the Compound-DMSO solution and DMSO as a control were dispensed into 2 wells. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast TM182-FoOS1 which had been prepared by dilution with a Glu-Ura-Leu medium as described above was dispensed, and cultured by allowing to stand at 30°C for 25 hours. In another microplate, as described above, as a control, each 100 μL of cell suspensions of the transformed budding yeast TM182-FoOS1 which had been prepared by dilution with a Gal-Ura-Leu medium was dispensed, and cultured at 30°C for 51 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

A degree of growth of each transformed budding yeast cultured in the presence of Compounds (1) to (7) is shown in Table 5. A degree of growth of the transformed budding yeast is expressed as a relative value in percentage, letting the absorbance at 600 nm at the concentration of the Compound of 0 ppm to be 100. It was confirmed that an inhibiting degree of growth of the transformed budding yeast TM182-FoOS1 by each test substance was grater than an inhibiting degree of growth of the transformed budding yeast AH22-FoOS1 by each test substance, and the transformed budding yeast TM182-FoOS1 was a transformed cell with the enhanced sensitivity to an antifungal

compound as compared with the transformed budding yeast AH22-FoOS1.

Table 5

	Degree of growth of budding yeast			
	AH22	AH22-FoOS1	TM182-FoOS1	
Test substance (final concentration)	Glu medium	Glu-Leu medium	Gal-Ura-Leu medium	Gul-Ura-Leu medium
Compoud (1) (6 ppm)	88	81	116	26
Compoud (2) (6 ppm)	91	91	87	55
Compoud (3) (6 ppm)	87	86	99	22
Compoud (4) (20 ppm)	96	90	104	20
Compoud (5) (20 ppm)	80	71	80	57
Compoud (6) (0.2 ppm)	92	69	99	7
Compoud (7) (0.2 ppm)	91	88	89	21

Example 14

Isolation of Mycospharella tritici StOS-1 gene

(1) Analysis of Mycospharella tritici StOS-1 gene fragment

The amplified DNA was purified from the reaction solution of PCR which had been performed using a primer pair 16 and using a cDNA of Mycospharella tritici as a template in Example 10 (4), using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. 3'A addition was performed on the purified DNA according to the method described in Example 11 (1). The 3'A-added DNA and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the instruction attached to the cloning vector, and this was introduced into Escherichia

coli JM109 (TaKaRa).

DNA was purified from the resulting Escherichia coli transformant by colony PCR using Ex Taq HS (TaKaRa). The PCR reaction solution (15 μL) was prepared by mixing 1.5 μL of 10×buffer, 2.25 μL of 10 mM dNTPs, 0.15 μL of Ex Taq HS, each 0.4 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 66 and a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 67, and 10.3 μL of sterilized distilled water, and adding a part of the Escherichia coli transformant colony thereto. PCR was performed under the amplifying conditions that this reaction solution was maintained at 97°C for 2 minutes and, thereafter, 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 97°C for 15 seconds, then, at 55°C for 15 seconds, then, at 72°C for 3 minutes. The amplified DNA was purified from the PCR reaction solution after temperature maintenance using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. A nucleotide sequence was analyzed using oligonucleotides consisting of nucleotide sequences represented by SEQ ID NOs: 29 and 54 as a primer and employing the purified DNA as a template according to the method described in Example 11 (1). As a result, a nucleotide sequence represented by base numbers 2241 to 3603 of the nucleotide sequence represented by SEQ ID NO: 56 was read.

(2) Analysis of full length Myçospharella tritici StOS-1 gene

A DNA having a nucleotide sequence extending toward to 5′ upstream region of a base number 2241 of the nucleotide sequence represented by SEQ ID NO: 56 was cloned using SMART RACE cDNA Amplification Kit (CLONTECH) according to the instruction attached to the kit. A reaction solution was prepared by mixing 1.0 μL of CDS-primer and 1.0 μL of SMART IIA Oligo attached to the kit into 3 μL (230ng) of total RNA prepared in Example 10 (2), a temperature was maintained at 70°C for 2 minutes, and maintained on ice for 2 minutes. To the reaction solution were added 2 μL of 5×First-Strand buffer attached to the kit, 1 μL of 20 mM DTT, 1 μL of 10 mM dNTP Mix and 1 μL of PowerScript Reverse Transcriptase, to mix them, and the mixture was maintained at 42°C for 1.5 hours. To the reaction solution after temperature maintenance was added 100 μL of Tricine-EDTA buffer attached to the kit, a temperature was maintained at 72°C for 7 minutes, thus 5′ RACE ready cDNA was prepared. PCR amplifying 5′ upstream region was performed using this 5′ RACE ready cDNA as a template and using KOD-plus-(TOYOBO). The PCR reaction solution was prepared by mixing 2.5 μL of 5′ RACE ready cDNA, 5.0 μL of 10×buffer, 5.0 μL of 2 mM dNTPs, 2.0 μL of 25 mM $MgSO_4$ and 1.0 μL of KOD-Plus, adding 5.0 μL of 10× Universal Primer A Mix attached to the kit and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 43 as primers, and adding sterilized

distilled water to a total amount of 50 μL. This reaction solution was maintained at 94°C for 2 minutes, and 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 68°C for 5 minutes. The amplified DNA was purified from the PCR reaction solution using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit, and then, 3'A addition was performed on the DNA according to the method described in Example 11(1). The 3'A-added DNA and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the instruction attached to the cloning vector, after that, which was introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA was purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence was analyzed using the resulting plasmid DNA as a template and using primers consisting of nucleotide sequences represented by SEQ ID NOs: 29, 54, and 59 to 61 according to the method described in Example 11(1). As a result, a nucleotide sequence represented by base numbers 1 to 2240 of the nucleotide sequence represented by SEQ ID NO: 56 was read.

Further, a DNA having a nucleotide sequence extending toward to the 3' downstream region from nucleotide number 3603 of the nucleotide sequence represented by SEQ ID NO: 56 was cloned. 1.0 μL of CDS-primer attached to the kit and 1.0 μL of sterilized distilled water were mixed into 3 μL (230ng) of the total RNA

prepared in Example 10 (2), the mixture was maintained at 70°C for 2 minutes, and maintained on ice for 2 minutes. 3′ RACE ready cDNA was prepared using the reaction solution as in preparation of 5′ RACE ready cDNA. PCR amplifying 3′ downstream region was performed using this 3′ RACE ready cDNA as a template. A PCR reaction solution was prepared by mixing 5.0 μL of 10× Advantage 2 buffer attached to the kit, 1.0 μL of 10 mM dNTP Mix and 1.0 μL of 50× Advantage 2 Polymerase Mix into 2.5 μL of 3′ RACE ready cDNA, adding 5.0 μL of 10×Universal Primer A Mix attached to the kit as a primer, and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 58, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution was subjected to repetition of 5 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 72°C for 4 minutes, further repetition of 5 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 70°C for 10 seconds, then, at 72°C for 4 minutes, further repetition of 25 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 68°C for 10 seconds, then, at 72°C for 4 minutes, followed by maintaining a temperature at 72°C for 7 minutes. The PCR reaction solution and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated to the vector according to the instruction attached to the kit, after that, which was introduced

into Escherichia coli JM109 (TaKaRa). A plasmid DNA was purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence was analyzed using the resulting plasmid DNA as a template, and using a primer consisting of the nucleotide sequence represented by any of SEQ ID NOs: 29 and 54, according to the method described in Example 11 (1). As a result, a nucleotide sequence represented by nucleotide numbers 3604 to 3924 of the nucleotide sequence represented by SEQ ID NO: 56 was read.

All analyzed nucleotide sequences were joined and, as a result, the nucleotide sequence represented by SEQ ID NO: 56 was obtained. The nucleotide sequence represented by SEQ ID NO: 56 consists of 3924 bases (including termination codon), and was a nucleotide sequence encoding 1307 amino acid residues (SEQ ID NO: 55). A molecular weight of a protein having the amino acid sequence represented by SEQ ID NO: 55 was calculated to be 143276 Da.

(3) Isolation of full length Mycospharella tritici StOS-1 gene

A DNA having a nucleotide sequence encoding an amino acid sequence of Mycospharella tritici StOS-1 (hereinafter, referred to as StOS-1 DNA in some cases) was amplified by PCR using the 5' RACE ready cDNA prepared in Example 14 (2) as a template. By performing a PCR using, as a primer, an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 64 and an oligonucleotide consisting of the nucleotide sequence

represented by SEQ ID NO: 65, a DNA having the nucleotide sequence represented by SEQ ID NO: 56 was amplified, according to the method described in Example 11 (3). After the reaction, a part of the PCR reaction solution was separated by 1% agarose gel electrophoresis, and stained with ethidium bromide. It was confirmed that about 4 kb of the DNA (StOS-1 DNA) was amplified.

Example 15

Construction of expression plasmid of Mycospharella tritici StOS-1 gene and preparation of transformed budding yeast

The StOS-1 DNA was cloned into a pCR2.1-TOPO cloning vector (Invitrogen). About 4 kb of the DNA (StOS-1 DNA) was purified from the PCR reaction solution prepared in Example 14 (3) using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. 3'A addition was performed on about 4 kb of the purified DNA (StOS-1 DNA) according to the method described in Example 11 (1). The 3'A-added about 4 kb DNA (StOS-1 DNA) and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the manual attached to the cloning vector to construct a plasmid pCRStOS1. A nucleotide sequence of the resulting plasmid was analyzed according to the method described in Example 11(1). As a primer, an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NOs: 29, 54, and 58 to 63 was used. As a result, the nucleotide sequence represented by SEQ ID NO: 56 was obtained, and it was

confirmed that the plasmid pCRStOS1 was a plasmid containing the StOS-1 DNA.

The StOS-1 DNA contained in the thus prepared plasmid pCRStOS1 was cloned into a shuttle vector p415ADH replicable in yeastand Escherichia coli to construct an expression plasmid. The plasmid pCRStOS1 was digested with restriction enzymes SpeI and HindIII and, on the other hand, the shuttle vector p415ADH was also digested with restriction enzymes SpeI and HindIII. Each of them was separated by 0.8% agarose gel electrophoresis, a part of the gel containing the StOS-1 DNA digested with SpeI and HindIII and the shuttle vector p415ADH digested with SpeI and HindIII was excised, and the StOS-1 DNA and the shuttle vector were recovered from the gel using QIAquick Gel Extraction Kit (QIAGEN) according to the attached manual. The StOS-1 DNA was inserted between SpeI site and HindIII site in the multicloning site of the shuttle vector using Ligation Kit Ver.2 (TaKaRa) according to the manual attached to the kit, whereby, an expression plasmid pADHStOS1 was constructed. A nucleotide sequence of the resulting expression plasmid was analyzed according to the method described in Example 11 (1). As a primer, an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NO: 58 to 65 was used. As a result, the nucleotide sequence represented by SEQ ID NO: 56 was obtained, and it was confirmed that the expression plasmid pADHStOS1 harbored a DNA having a nucleotide sequence encoding an amino

acid sequence of StOS-1.

The prepared expression plasmid pADHStOS1 was introduced into budding yeast AH22 strain and TM182 strain according to the method described in Example 2. By utilizing the disappearance of leucine auxotrophy in the resulting transformed budding yeast, the transformed budding yeast AH22 strain (AH22-StOS1) was selected on a Glu-Leu agar medium, and the transformed budding yeast TM182 strain (TM182-StOS1) was selected on a Gal-Ura-Leu agar medium. It was confirmed that the resulting TM182-StOS1 grows even when transplanted to a Glu-Ura-Leu medium.

Example 16

Antifungal compound sensitivity test of transformed budding yeast TM182-StOS1

The transformed budding yeast AH22-StOS1 prepared in Example 15 was cultured while shaking at 30°C for 18 hours in a Glu-Leu medium. As a control, the AH22 strain was similarly cultured while shaking at 30°C for 18 hours in a Glu medium. The absorbance at 600 nm of each grown transformed budding yeast in a cell suspension was measured, and cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast AH22-StOS1 was diluted 50-fold with a Glu-Leu medium, and a cell

suspension in which the aforementioned cell suspension of the AH22 strain was diluted 50-fold with a Glu medium were prepared.

A solution in which each of Compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 6 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 2000 ppm, and a solution in which Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 1.0 μL per well of the Compound solution and DMSO as a control were dispensed. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast AH22-StOS1 which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30 °C for 28 hours. In another microplate, each 100 μL of cell suspensions of the control yeast AH22 strain which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30°C for 24.5 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

Similarly, the transformed budding yeast TM182-StOS1 prepared in Example 15 was cultured at 30°C for 18 hours in a Glu-Ura-Leu medium. The absorbance at 600 nm of the grown transformed budding yeast in a cell suspension was measured, and a cell suspension diluted with sterilized distilled water

to the absorbance of 0.1 was prepared. Further, a cell suspension in which the transformed budding yeast TM182-StOS1 was diluted 50-fold with a Glu-Ura-Leu medium and, as a control, a cell suspension in which the yeast was diluted 50-fold with a Gal-Ura-Leu medium were prepared.

A solution in which each of Compounds (1) to (3) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 6 ppm, a solution in which each of Compounds (4) and (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 2000 ppm, and a solution in which each of Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 20 ppm were prepared, and two microplates were prepared in which each 2.0 μL per well of the Compound-DMSO solution and DMSO as a control were dispensed into 2 wells. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast TM182-StOS1 which had been prepared by dilution with a Glu-Ura-Leu medium as described above was dispensed, and cultured by allowing to stand at 30°C for 26.5 hours. In another microplate, as described above, as a control, each 100 μL of cell suspensions of the transformed budding yeast TM182-StOS1 which had been prepared by dilution with a Gal-Ura-Leu medium was dispensed, and cultured at 30°C for 49.5 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

A degree of growth of each transformed budding yeast

cultured in the presence of Compounds (1) to (7) is shown in Table 6. A degree of growth of the transformed budding yeast is expressed as a relative value in percentage, letting the absorbance at 600 nm at the concentration of the Compound of 0 ppm to be 100. It was confirmed that an inhibiting degree of growth of the transformed budding yeast TM182-StOS1 by each test substance was grater than an inhibiting degree of growth of the transformed budding yeast AH22-StOS1 by each test substance, and the transformed budding yeast TM182-StOS1 was a transformed cell with the enhanced sensitivity to an antifungal compound as compared with the transformed budding yeast AH22-StOS1.

Table 6

	Degree of growth of budding yeast			
	AH22	AH22-StOS1	TM182-StOS1	
Test substance (final concentration)	Glu medium	Glu-Leu medium	Gal-Ura-Leu medium	Gul-Ura-Leu medium
Compoud (1) (0.6 ppm)	99	101	101	67
Compoud (2) (0.6 ppm)	94	100	97	23
Compoud (3) (0.6 ppm)	96	98	94	19
Compoud (4) (20 ppm)	96	91	99	7
Compoud (5) (20 ppm)	80	76	74	6
Compoud (6) (0.2 ppm)	92	93	97	6
Compoud (7) (0.2 ppm)	91	91	91	9

Example 17

Isolation of Thanatephorus cucumeris RsOS-1 gene

(1) Analysis of Thanatephorus cucumeris RsOS-1 gene fragment

The amplified DNA was purified from the reaction solution of PCR which had been performed using a primer pair 3 and using a cDNA of Thanatephorus cucumeris as a template in Example 10 (5), using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. 3'A addition was performed on the purified DNA according to the method described in Example 11 (1). The 3'A-added DNA and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the instruction attached to the cloning vector, and this was introduced into Escherichia coli JM109 (TaKaRa).

DNA was purified from the resulting Escherichia coli transformant by colony PCR using Ex Taq HS (TaKaRa). The PCR reaction solution (15 μL) was prepared by mixing 1.5 μL of 10×buffer, 2.25 μL of 10 mM dNTPs, 0.15 μL of Ex Taq HS, each 0.4 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 28 and a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 29, and 10.3 μL of sterilized distilled water, and adding a part of the Escherichia coli transformant colony thereto. PCR was performed under the amplifying conditions that this reaction solution was maintained at 97°C for 2 minutes and, thereafter, 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 97°C for 15 seconds, then, at 55°C for 15 seconds, then, at

72°C for 3 minutes. The amplified DNA was purified from the PCR reaction solution after temperature maintenance using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. A nucleotide sequence was analyzed using oligonucleotides consisting of nucleotide sequences represented by SEQ ID NOs: 28 and 29 as a primer and employing the purified DNA as a template according to the method described in Example 11 (1). As a result, a nucleotide sequence represented by base numbers 2838 to 3165 of the nucleotide sequence represented by SEQ ID NO: 69 was read.

(2) Analysis of full length Thanatephorus cucumeris RsOS-1 gene

A DNA having a nucleotide sequence extending toward to 3′ downstream region of a base number 3165 of the nucleotide sequence represented by SEQ ID NO: 69 was cloned using SMART RACE cDNA Amplification Kit (CLONTECH) according to the instruction attached to the kit. A reaction solution was prepared by mixing 1.0 μL of CDS-primer and 1.0 μL of sterilized distilled water attached to the kit into 3 μL (253ng) of total RNA prepared in Example 10 (3), a temperature was maintained at 70°C for 2 minutes, and maintained on ice for 2 minutes. To the reaction solution were added 2 μL of 5×First-Strand buffer attached to the kit, 1 μL of 20 mM DTT, 1 μL of 10 mM dNTP Mix and 1 μL of PowerScript Reverse Transcriptase, to mix them, and the mixture was maintained at 42°C for 1.5 hours. To the reaction solution after temperature maintenance was added 100 μL of

Tricine-EDTA buffer attached to the kit, a temperature was maintained at 72°C for 7 minutes, thus 3′ RACE ready cDNA was prepared. PCR amplifying 3′ downstream region was performed using this 3′ RACE ready cDNA as a template and using KOD-plus-(TOYOBO). The PCR reaction solution was prepared by mixing 2.5 μL of 3′ RACE ready cDNA, 5.0 μL of 10×buffer, 5.0 μL of 2 mM dNTPs, 2.0 μL of 25 mM $MgSO_4$ and 1.0 μL of KOD-Plus, adding 5.0 μL of 10× Universal Primer A Mix attached to the kit and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 70 as primers, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution was maintained at 94°C for 2 minutes, and 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 68°C for 6 minutes. The amplified DNA was purified from the PCR reaction solution using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit, and then, 3′A addition was performed on the DNA according to the method described in Example 11(1). The 3′A-added DNA and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the instruction attached to the cloning vector, after that, which was introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA was purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence was analyzed using the resulting plasmid

DNA as a template and using primers consisting of nucleotide sequences represented by SEQ ID NOs: 28, 29, and 73 to 76 according to the method described in Example 11(1). As a result, a nucleotide sequence represented by base numbers 3119 to 4317 of the nucleotide sequence represented by SEQ ID NO: 69 was read.

Further, a DNA having a nucleotide sequence extending toward to the 5′ upstream region from nucleotide number 2838 of the nucleotide sequence represented by SEQ ID NO: 69 was cloned. 1.0 μL of CDS-primer attached to the kit and 1.0 μL of SMART IIA Oligo were mixed into 3 μL (253ng) of the total RNA prepared in Example 10 (3), the mixture was maintained at 70°C for 2 minutes, and maintained on ice for 2 minutes. 5′ RACE ready cDNA was prepared using the reaction solution as in preparation of 3′ RACE ready cDNA. PCR amplifying 5′ upstream region was performed using this 5′ RACE ready cDNA as a template and using KOD-plus-(TOYOBO). The PCR reaction solution was prepared by mixing 2.5 μL of 5′ RACE ready cDNA, 5.0 μL of 10×buffer, 5.0 μL of 2 mM dNTPs, 2.0 μL of 25 mM $MgSO_4$ and 1.0 μL of KOD-Plus, adding 5.0 μL of 10× Universal Primer A Mix attached to the kit and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 71 as primers, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution was maintained at 94°C for 2 minutes, and 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 68°C

for 6 minutes. Using the resulting PCR reaction solution as a template, the PCR reaction solution for a futher PCR was prepared by adding 5.0 μL of 10×buffer, 5.0 μL of 2 mM dNTPs, 2.0 μL of 25 mM $MgSO_4$ and 1.0 μL of KOD-Plus, 1.0 μL of 10 μM Nested universal primer attached to the kit and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 72 as primers, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution was maintained at 94°C for 2 minutes, and 20 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 68°C for 6 minutes. The PCR reaction solution and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated to the vector according to the instruction attached to the kit, after that, which was introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA was purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence was analyzed using the resulting plasmid DNA as a template, and using a primer consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28, 29, and 77 to 82, according to the method described in Example 11 (1). As a result, a nucleotide sequence represented by nucleotide numbers 1 to 3042 of the nucleotide sequence represented by SEQ ID NO: 69 was read.

All analyzed nucleotide sequences were joined and, as a result, the nucleotide sequence represented by SEQ ID NO: 69

was obtained. The nucleotide sequence represented by SEQ ID NO: 69 consists of 4317 bases (including termination codon), and was a nucleotide sequence encoding 1438 amino acid residues (SEQ ID NO: 68). A molecular weight of a protein having the amino acid sequence represented by SEQ ID NO: 68 was calculated to be 155296 Da.

(3) Isolation of full length Thanatephorus cucumeris RsOS-1 gene

A DNA having a nucleotide sequence encoding an amino acid sequence of Thanatephorus cucumeris RsOS-1 (hereinafter, referred to as RsOS-1 DNA in some cases) was amplified by PCR using a cDNA of Thanatephorus cucumeris prepared in Example 10 (5) as a template. By performing a PCR using, as a primer, an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 85 and an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 86, a DNA having the nucleotide sequence represented by SEQ ID NO: 69 was amplified, according to the method described in Example 11 (3). After the reaction, a part of the PCR reaction solution was separated by 1% agarose gel electrophoresis, and stained with ethidium bromide. It was confirmed that about 4 kb of the DNA (RsOS-1 DNA) was amplified.

Example 18

Construction of expression plasmid of Thanatephorus cucumeris RsOS-1 gene and preparation of transformed budding yeast

The RsOS-1 DNA was cloned into a pCR2.1-TOPO cloning vector (Invitrogen). About 4 kb of the DNA (RsOS-1 DNA) was purified from the PCR reaction solution prepared in Example 17 (3) using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. 3'A addition was performed on about 4 kb of the purified DNA (StOS-1 DNA) according to the method described in Example 11 (1). The 3'A-added about 4 kb DNA (RsOS-1 DNA) and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the manual attached to the cloning vector to construct a plasmid pCRRsOS1. A nucleotide sequence of the resulting plasmid was analyzed according to the method described in Example 11(1). As a primer, an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28, 29, 70 to 73, 75, 77, 78, and 81 to 84 was used. As a result, the nucleotide sequence represented by SEQ ID NO: 69 was obtained, and it was confirmed that the plasmid pCRRsOS1 was a plasmid containing the RsOS-1 DNA.

The RsOS-1 DNA contained in the thus prepared plasmid pCRRsOS1 was cloned into a shuttle vector p415ADH replicable in yeastand Escherichia coli to construct an expression plasmid. The plasmid pCRRsOS1 was digested with restriction enzymes SpeI and HindIII and, on the other hand, the shuttle vector p415ADH was also digested with restriction enzymes SpeI and HindIII. Each of them was separated by 0.8% agarose gel electrophoresis, a part of the gel containing the RsOS-1 DNA digested with SpeI

and HindIII and the shuttle vector p415ADH digested with SpeI and HindIII was excised, and the RsOS-1 DNA and the shuttle vector were recovered from the gel using QIAquick Gel Extraction Kit (QIAGEN) according to the attached manual. The RsOS-1 DNA was inserted between SpeI site and HindIII site in the multicloning site of the shuttle vector using Ligation Kit Ver.2 (TaKaRa) according to the manual attached to the kit, whereby, an expression plasmid pADHRsOS1 was constructed. A nucleotide sequence of the resulting expression plasmid was analyzed according to the method described in Example 11 (1). As a primer, an oligonucleotide consisting of the nucleotide sequence represented by any of SEQ ID NO: 70 to 73, 75, 77, 78, 81 to 84, 87 and 88 was used. As a result, the nucleotide sequence represented by SEQ ID NO: 69 was obtained, and it was confirmed that the expression plasmid pADHRsOS1 harbored a DNA having a nucleotide sequence encoding an amino acid sequence of RsOS-1.

The prepared expression plasmid pADHRsOS1 was introduced into budding yeast AH22 strain and TM182 strain according to the method described in Example 2. By utilizing the disappearance of leucine auxotrophy in the resulting transformed budding yeast, the transformed budding yeast AH22 strain (AH22-RsOS1) was selected on a Glu-Leu agar medium, and the transformed budding yeast TM182 strain (TM182-RsOS1) was selected on a Gal-Ura-Leu agar medium. It was confirmed that the resulting TM182-RsOS1 grows even when transplanted to a

Glu-Ura-Leu medium.

Example 19

Antifungal compound sensitivity test of transformed budding yeast TM182-RsOS1

The transformed budding yeast AH22-RsOS1 prepared in Example 18 was cultured while shaking at 30°C for 18 hours in a Glu-Leu medium. As a control, the AH22 strain was similarly cultured while shaking at 30°C for 18 hours in a Glu medium. The absorbance at 600 nm of each grown transformed budding yeast in a cell suspension was measured, and cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast AH22-RsOS1 was diluted 50-fold with a Glu-Leu medium, and a cell suspension in which the aforementioned cell suspension of the AH22 strain was diluted 50-fold with a Glu medium were prepared.

A solution in which each of Compounds (1) to (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 600 ppm, and a solution in which Compounds (6) and (7) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 60 ppm were prepared, and two microplates were prepared in which each 1.0 μL per well of the Compound solution and DMSO as a control were dispensed. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast AH22-RsOS1 which

had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30 °C for 29.8 hours. In another microplate, each 100 μL of cell suspensions of the control yeast AH22 strain which had been prepared by dilution as described above was dispensed, and cultured by allowing to stand at 30°C for 24.8 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

Similarly, the transformed budding yeast TM182-RsOS1 prepared in Example 18 was cultured at 30°C for 18 hours in a Glu-Ura-Leu medium. The absorbance at 600 nm of the grown transformed budding yeast in a cell suspension was measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the transformed budding yeast TM182-RsOS1 was diluted 50-fold with a Glu-Ura-Leu medium. As a control, the transformed budding yeast TM182-RsOS1 was cultured at 30°C for 18 hours in a Gal-Ura-Leu medium. The absorbance at 600 nm of the grown transformed budding yeast in a cell suspension was measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 was prepared. Further, a cell suspension in which the transformed budding yeast TM182-RsOS1 was diluted 50-fold with a Gal-Ura-Leu medium.

A solution in which each of Compounds (1) to (5) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 600 ppm, and a solution in which each of Compounds (6) and (7)

was dissolved in dimethylsulfoxide (DMSO) to the concentration of 60 ppm were prepared, and two microplates were prepared in which each 2.0 μL per well of the Compound-DMSO solution and DMSO as a control were dispensed into 2 wells. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast TM182-RsOS1 which had been prepared by dilution with a Glu-Ura-Leu medium as described above was dispensed, and cultured by allowing to stand at 30°C for 26.8 hours. In another microplate, as described above, as a control, each 100 μL of cell suspensions of the transformed budding yeast TM182-RsOS1 which had been prepared by dilution with a Gal-Ura-Leu medium was dispensed, and cultured at 30°C for 42.5 hours. After culturing, the absorbance at 600 nm of each well was measured with a microplate reader.

A degree of growth of each transformed budding yeast cultured in the presence of Compounds (1) to (7) is shown in Table 7. A degree of growth of the transformed budding yeast is expressed as a relative value in percentage, letting the absorbance at 600 nm at the concentration of the Compound of 0 ppm to be 100. It was confirmed that an inhibiting degree of growth of the transformed budding yeast TM182-RsOS1 by each test substance was grater than an inhibiting degree of growth of the transformed budding yeast AH22-RsOS1 by each test substance, and the transformed budding yeast TM182-RsOS1 was a transformed cell with the enhanced sensitivity to an antifungal

compound as compared with the transformed budding yeast AH22-RsOS1.

Table 7

	Degree of growth of budding yeast			
	AH22	AH22-RsOS1	TM182-RsOS1	
Test substance (final concentration)	Glu medium	Glu-Leu medium	Gal-Ura-Leu medium	Gul-Ura-Leu medium
Compoud (1) (6.0 ppm)	88	103	108	15
Compoud (2) (6.0 ppm)	92	101	96	11
Compoud (3) (6.0 ppm)	82	101	101	27
Compoud (4) (6.0 ppm)	83	89	88	17
Compoud (5) (6.0 ppm)	78	85	101	9
Compoud (6) (0.6 ppm)	79	79	100	12
Compoud (7) (0.6 ppm)	85	101	99	31

Example 20

Isolation of a gene of the present histidine kinase of Phytophthora infestans (hereinafter, referred to PiOS-1 gene)

(1) Analysis of Phytophthora infestans PiOS-1 gene fragment

The amplified DNA was purified from the reaction solution of PCR which had been performed using a primer pair 6 and using a cDNA of Phytophthora infestans as a template in Example 10(5), using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit. 3'A addition was performed on the purified DNA according to the method described in Example 11 (1). The 3'A added DNA and the pCR2.1-TOPO cloning vector (Invitrogen) were ligated according to the instruction attached

to the cloning vector, after that, which was introduced into Escherichia coli JM109 (TaKaRa).

A DNA was amplified from the resulting Escherichia coli transformant by colony PCR using Ex Taq HS (TaKaRa). The PCR reaction solution (15 μL) was prepared by mixing 1.5 μL of 10×buffer, 2.25 μL of 10 mM dNTPs, 0.15 μL of Ex Taq HS, each 0.4 μL of a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 28 and a 10 μM solution of an oligonucleotide consisting of the nucleotide sequence represented by SEQ ID NO: 29, and 10.3 μL of sterilized distilled water, and adding a part of the Escherichia coli transformant colony thereto. PCR was performed under the amplifying conditions that this reaction solution was maintained at 97°C for 2 minutes, and 35 cycles of incubation were repeated, each cycle comprising maintaining a temperature at 97°C for 15 seconds, then, at 55°C for 15 seconds, further, at 72°C for 3 minutes. The amplified DNA was purified from the PCR reaction solution after temperature maintenance using QIAquick PCR purification Kit (QIAGEN) according to the manual attached to the kit. A nucleotide sequence was analyzed using the purified DNA as a template and using oligonucleotides consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28 and 29 as primers according to the method described in Example 11(1). As a result, a nucleotide sequence represented by SEQ ID NO: 89 containing a nucleotide sequence of an oligonucleotide used

as a primer pair 6 was read.

(2) Analysis of full length Phytophthora infestans PiOS-1 gene

A DNA having a nucleotide sequence extending toward to 5′ upstream region of a nucleotide sequence represented by SEQ ID NO: 89 is cloned using SMART RACE cDNA Amplification Kit (CLONTECH) according to the instruction attached to the kit. A reaction solution is prepared by mixing 1.0 μL of CDS-primer attached to the kit and 1.0 μL of SMART IIA Oligo into 3 μL (200ng) of the total RNA prepared in Example 10 (4), a temperature is maintained at 70°C for 2 minutes, and is maintained on ice for 2 minutes. To the reaction solution are added 2 μL of 5×First-Strand buffer attached to the kit, 1 μL of 20 mM DTT, 1 μL of 10 mM dNTP Mix and 1 μL of PowerScript Reverse Transcriptase to mix them, and the mixture is maintained at 42°C for 1.5 hours. To the reaction solution after temperature maintenance is added 100 μL of Tricine-EDTA buffer attached to the kit, a temperature is maintained at 72°C for 7 minutes, and 5′ RACE ready cDNA is prepared. PCR amplifying 5′ upstream region is performed using this 5′ RACE ready cDNA as a template and using KOD-plus-(TOYOBO). The PCR reaction solution is prepared by mixing 2.5 μL of 5′ RACE ready cDNA, 5.0 μL of 10×buffer, 5.0 μL of 2 mM dNTPs, 2.0 μL of 25 mM $MgSO_4$ and 1.0 μL of KOD-Plus-, adding 5.0 μL of 10×Universal primer A Mix attached to the kit as a primer and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of 20 to 30 bases selected from complementary sequences of the

nucleotide sequence represented by SEQ ID NO: 89, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution is maintained at 94°C for 2 minutes, and further 35 cycles of incubation are repeated, each cycle comprising maintaining a temperature at 94°C for 15 seconds, then, at 68°C for 5 minutes. The amplified DNA is purified from the PCR reaction solution using QIAquick PCR Purification Kit (QIAGEN) according to the instruction attached to the kit, and 3′ A addition is performed on the DNA according to the method described in Example 11 (1). 3′ A added DNA and the pCR2.1-TOPO cloning vector (Invitrogen) are ligated according to the instruction attached to the cloning vector, after that, which is introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA is purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence is analyzed using the resulting plasmid DNA as a template and using primers consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28, 29, and the like according to the method described in Example 11(1). As a result, a nucleotide sequence of the 5′-terminal region including a translation initiation codon of an os-1 homologous gene of Phytophthora infestans, that is, gene of Phytophthora infestans encoding osmosensing histidine kinase having no transmembrane region (PiOS1) can be read.

Further, a DNA having a nucleotide sequence extending to 3′ downstream region of the nucleotide sequence represented by

SEQ ID NO: 89 is cloned. 1.0 μL of CDS-primer attached to the kit and 1.0 μL of sterilized distilled water are mixed into 3 μL (200ng) of the total RNA prepared in Example 10 (4), a temperature is maintained at 70°C for 2 minutes, and is maintained on ice for 2 minutes. 3′ RACE ready cDNA is prepared using the reaction solution as in preparation of 5′ RACE ready cDNA. PCR amplifying 3′ downstream region is performed using this 3′ RACE ready cDNA as a template. The PCR reaction solution is prepared by mixing 5.0 μL of 10×Advantage 2 buffer attached to the kit, 1.0 μL of 10 mM dNTP Mix and 1.0 μL of 50×Advantage 2 polymerase Mix into 2.5 μL of 5′ RACE ready cDNA, adding 5.0 μL of 10×Universal Primer A Mix attached to the kit, and 1.0 μL of a 10 μM solution of an oligonucleotide consisting of 20 to 30 bases selected from the nucleotide sequence represented by SEQ ID NO: 89 as primers, and adding sterilized distilled water to a total amount of 50 μL. This reaction solution is subjected to repetition of 5 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 72°C for 4 minutes, further repetition of 5 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 70°C for 10 seconds then, at 72°C for 4 minutes, further repetition of 25 cycles of incubation, each cycle comprising maintaining a temperature at 94°C for 5 seconds, then, at 68°C for 10 seconds, then, at 72°C for 4 minutes, followed by maintaining a temperature at 72°C for 7 minutes. The PCR reaction solution and the

pCR2.1-TOPO cloning vector (Invitrogen) are ligated according to the instruction attached to the cloning vector, after that, which is introduced into Escherichia coli JM109 (TaKaRa). A plasmid DNA is purified from the resulting Escherichia coli transformant using QIAprep Spin Miniprep Kit (QIAGEN). A nucleotide sequence is analyzed using the resulting plasmid DNA as a template and using primers consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28, 29, and the like according to the method described in Example 11 (1). As a result, a nucleotide sequence of the 3'-terminal region including a translation termination codon of a Phytophthora infestans PiOS1 gene is read.

By ligating all analyzed nucleotide sequences, full nucleotide sequence of Phytophthora infestans PiOS-1 gene including nucleotide sequence represented by SEQ ID NO: 89 is confirmed.

(3) Isolation of full length Phytophthora infestans PiOS1 gene

A DNA having a nucleotide sequence encoding an amino acid sequence of Phytophthora infestans PiOS1 (hereinafter, referred to as PiOS-1 DNA) is amplified by PCR using the cDNA prepared in Example 10 (4) as a template. Using as primers an oligonucleotide comprising a nucleotide sequence in which a nucleotide sequence ACGACAGT is added to the 5'-terminal end of a nucleotide sequence from the 5'-terminal end to the 20th base including the initiation codon of a nucleotide sequence

of Phytophthora infestans PiOS-1 gene obtained in Example 20 (2), and an oligonucleotide having a nucleotide sequence complementary to a nucleotide sequence in which a nucleotide sequence AAGCTTCAG is added to the 3'-terminal end of a nucleotide sequence of from the 3'-terminal end to the 20th base including the termination codon of a nucleotide sequence of Phytophthora infestans PiOS-1 gene obtained in Example 20 (2), a PCR is performed according to the method described in Example 11 (3). DNA containing a nucleotide sequence encoding an amino acid sequence of Phytophthora infestans PiOS-1, and having a recognition sequence of a restriction enzyme SpeI immediately before an initiation codon, and having a recognition sequence of a restriction enzyme HindIII immediately after a termination codon is amplified. A part of the PCR reaction solution after the reaction is separated by 1% agarose gel electrophoresis, and stained with ethidium bromide. It is confirmed that the about 4 kb PiOS-1 DNA is amplified.

Example 21

Construction of expression plasmid of Phytophthora infestans PiOS-1 gene and preparation of transformed budding yeast

The PiOS-1 DNA is cloned into the pCR2.1-TOPO cloning vector (Invitrogen). An about 4 kb DNA (PiOS-1 DNA) is purified from the PCR reaction solution prepared in Example 20 (3) using QIAquick PCR Purification Kit (QIAGEN) according to the manual

attached to the kit. 3'A addition is performed on the about 4 kb purified DNA according to the method described in Example 11 (3). The about 4 kb 3'A-added DNA (PiOS-1 DNA) and the pCR2.1-TOPO cloning vector (Invitrogen) are ligated according to the instruction attached to the cloning vector, whereby, the plasmid pCRPiOS1 is constructed. A nucleotide sequence of the resulting plasmid is analyzed by the method described in Example 11 (1). As a primer, oligonucleotides consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28 and 29 are used. As a result, it is confirmed that the plasmid pCRPiOS1 is a plasmid harboring PiOS-1 DNA containing the nucleotide sequence represented by SEQ ID NO: 89.

The Phytophthora infestans PiOS-1 gene contained in the thus prepared plasmid pCR PiOS1 is cloned into a shuttle vector p415ADH replicable in yeast and Escherichia coli, whereby, an expression plasmid is constructed. The plasmid pCRPiOS1 is digested with restriction enzymes SpeI and HindIII and, on the other hand, the shuttle vector p415ADH is also digested with restriction enzymes SpeI and HindIII. These are separated by 0.8% agarose gel electrophoresis, respectively, thereafter, a part of the gel containing the PiOS-1 DNA digested with restriction enzymes SpeI and HindIII and the shuttle vector p415ADH digested with SpeI and HindIII is excised, and the PiOS-1 DNA and the shuttle vector are recovered from the gel using QIAquick Gel Extraction Kit (QUAGEN) according to the manual

attached to the kit. Using Ligation Kit Ver.2 (TaKaRa) according to the manual attached to the kit, the PiOS-1 DNA is inserted between SpeI site and HindIII site in the multicloning site of the shuttle vector, whereby, the expression plasmid pADHPiOS1 is constructed. A nucleotide sequence of the resulting expression plasmid is analyzed according to the method described in Example 11 (1). As a primer, oligonucleotides consisting of the nucleotide sequence represented by any of SEQ ID NOs: 28 and 29 are used. As a result, it is confirmed that the expression plasmid pADHPiOS1 is a plasmid harboring the PiOS-1 DNA containing the nucleotide sequence represented by SIQ ID NO: 89.

The prepared expression plasmid pADH PiOS1 is gene-introduced into budding yeast AH22 strain and TM182 strain according to the method described in Example 2. By utilizing disappearance of leucine auxotrophy in the resulting transformed budding yeast, the transformed budding yeast AH22 strain (AH22-PiOS1) is selected on a Glu-Leu agar medium, and the transformed budding yeast TM182 strain (TN182-PiOS1) is selected on a Gal-Ura-Leu agar medium. It is confirmed that the resulting TM182-PiOS1 grows even when transplanted to a Glu-Ura-Leu medium.

Example 22

Antifungal compound sensitivity test of transformed budding yeast TM182-PiOS1

The transformed budding yeast AH22-PiOS1 prepared in Example 21 is cultured while shaking at 30°C in a Glu-Leu medium. As a control, the AH22 strain is similarly cultured while shaking at 30°C in a Glu medium. The absorbance at 600 nm of each of grown transformed budding yeasts in a cell suspension is measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 is prepared. Further, a cell suspension in which the aforementioned suspension of the transformed budding yeast AH22-PiOS1 is diluted 50-fold with a Glu-Leu medium, and a cell suspension in which the aforementioned suspension of the AH22 strain is diluted 50-fold with a Glu medium are prepared.

A solution in which each of Compounds (1) to (7) is dissolved in dimethylsulfoxide (DMSO) is prepared, and two microplates are prepared in which each 1.0 μL per well of each of the Compound Solution and DMSO as a control are dispensed into two wells. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast AH22-PiOS1 which has been prepared by dilution as described above is dispensed, and is cultured by allowing to stand at 30°C. In another microplate, each 100 μL of cell suspensions of the control yeast AH22 strain which has been prepared by dilution as described above is dispensed, and is cultured by allowing to stand at 30°C. After culturing, the absorbance at 600 nm of each well is measured with a microplate reader.

Similarly, the transformed budding yeast TM182-PiOS1

prepared in Example 21 is cultured at 30°C in a Glu-Ura-Leu medium. The absorbance at 600 nm of a cell suspension of the grown transformed budding yeast is measured, and a cell suspension diluted with sterilized distilled water to the absorbance of 0.1 is prepared. Further, a cell suspension in which the aforementioned cell suspension of the transformed budding yeast TM182-PiOS1 is diluted 50-fold with a Glu-Ura-Leu medium and, as a control, a cell suspension in which the aforementioned cell suspension is diluted 50-fold with a Gal-Ura-Leu medium are prepared.

A solution in which each of Compounds (1) to (7) is dissolved in dimethylsulfoxide (DMSO) is dissolved is prepared, and two microplates are prepared in which each 1.0 μL per well of the Compound solution and DMSO as a control are dispensed. In one microplate among them, each 100 μL of cell suspensions of the transformed budding yeast TM182-PiOS1 which has been prepared by dilution with a Glu-Ura-Leu medium as described above is dispensed, and is cultured by allowing to stand at 30°C. In another microplate as described above, as a control, each 100 μL of cell suspensions of the transformed budding yeast TM182-PiOS1 which has been prepared by dilution with a Gal-Ura-Leu medium is dispensed, and is cultured by allowing to stand at 30°C. After culturing, the absorbance at 600 nm of each well is measured with a microplate reader.

It is confirmed that an inhibiting degree of growth of

the transformed budding yeast TM182-PiOS1 by each test substance is greater than an inhibiting degree of growth of the transformed budding yeast AH22-PiOS1 by each test substance, and the transformed budding yeast TM182-PiOS1 is a transformed cell with the enhanced sensitivity to an antifungal compound as compared with the transformed budding yeast AH2-PiOS1.

The compositions of media used in the present invention are described below.

(a) Glu-medium

Becto-yeast nitrogen base without amino acids 6.7 g, Glucose 20 g, Drop-out mix(1) 2.0 g, Distilled water 1000 ml

(b) Glu-Leu medium

Bacto-yeast nitrogen base without amino acids 6.7 g, Glucose 20 g, Drop-out mix(2) 2.0 g, Distilled water 1000 ml

(c) Glu-Ura-Leu medium

Bacto-yeast nitrogen base without amino acids 6.7 g, Glucose 20 g, Drop-out mix (3) 2.0 g,

Distilled water 1000 ml

(d) Gal-Ura-Leu medium

Bacto-yeast nitrogen base without amino acids 6.7 g,

Galactose 20 g Drop-out mix (3) 2.0 g,

Distilled water 1000 ml

Drop-out mix (1):

Adenine 0.5 g, Lysine 2.0 g, Alanine 2.0 g, Methionine

2.0 g, Arginine 2.0 g, para-Aminobenzoic acid 0.2g, Asparagine 2.0 g. Phenylalanine 2.0 g, Aspartic acid 2.0 g, Proline 2.0 g, Cysteine 2.0 g, Serine 2.0 g, Glutamine 2.0 g, Threonine 2.0 g, Glutamic acid 2.0 g, Tryptophan 2.0 g, Glycine 2.0 g, Tyrosine 2.0 g, Histidine 2.0 g, Valine 2.0 g, Inositol 2.0 g, Isoleucine 2.0 g, Uracil 2.0 g, Leucine 10.0 g, Distilled water 1000 ml

Drop-out mix (2): Drop-out mix (1) except for leucine (10.0 g)

Drop-out mix (3): Drop-out mix (1) except for uracil (2.0 g) and leucine (10.0 g)

(e) Glu-agar medium

Solid medium in which 2%(W/V) agar is added to a medium (a)

(f) Glu-Leu agar medium

Solid medium in which 2% (W/V) agar is added to a medium (b)

(g) Glu-Ura-Leu agar medium

Solid medium in which 2% (W/V) agar is added to a medium (c)

(h) Gal-Ura-Leu agar medium

Solid medium in which 2%(W/V) agar is added to a medium (d)

Free text in Sequence Listing

SEQ ID NO:3

Designed oligonucleotide primer for PCR

SEQ ID NO:4

Designed oligonucleotide primer for PCR

SEQ ID NO:5

Designed oligonucleotide primer for sequencing

SEQ ID NO:6

Designed oligonucleotide primer for sequencing

SEQ ID NO:7

Designed oligonucleotide primer for sequencing

SEQ ID NO:8

Designed oligonucleotide primer for sequencing

SEQ ID NO:9

Designed oligonucleotide primer for sequencing

SEQ ID NO:10

Designed oligonucleotide primer for sequencing

SEQ ID NO:11

Designed oligonucleotide primer for sequencing

SEQ ID NO:12

Designed oligonucleotide primer for sequencing

SEQ ID NO:15

Designed oligonucleotide primer for PCR

SEQ ID NO:18

Designed oligonucleotide primer for PCR

SEQ ID NO:19

Designed oligonucleotide primer for PCR

SEQ ID NO:20

Designed oligonucleotide primer for sequencing

SEQ ID NO:21

Designed oligonucleotide primer for sequencing

SEQ ID NO:22

Designed oligonucleotide primer for sequencing

SEQ ID NO:23

Designed oligonucleotide primer for sequencing

SEQ ID NO:24

Designed oligonucleotide primer for sequencing

SEQ ID NO:25

Designed oligonucleotide primer for sequencing

SEQ ID NO:26

Designed oligonucleotide primer for sequencing

SEQ ID NO:27

Designed oligonucleotide primer for sequencing

SEQ ID NO:28

Designed oligonucleotide primer for sequencing

SEQ ID NO:29

Designed oligonucleotide primer for sequencing

SEQ ID NO:30

Designed oligonucleotide primer for PCR

SEQ ID NO:31

Designed oligonucleotide primer for PCR

SEQ ID NO:32

Designed oligonucleotide primer for PCR

SEQ ID NO:33

Designed oligonucleotide primer for PCR

SEQ ID NO:34

Designed oligonucleotide primer for PCR

SEQ ID NO:35

Designed oligonucleotide primer for PCR

SEQ ID NO:36

Designed oligonucleotide primer for PCR

SEQ ID NO:37

Designed oligonucleotide primer for PCR

SEQ ID NO:38

Designed oligonucleotide primer for PCR

SEQ ID NO:39

Designed oligonucleotide primer for PCR

SEQ ID NO:40

Designed oligonucleotide primer for PCR

SEQ ID NO:43

Designed oligonucleotide primer for PCR

SEQ ID NO:44

Designed oligonucleotide primer for PCR

SEQ ID NO:45

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:46

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:47

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:48

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:49

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:50

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:51

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:52

Designed oligonucleotide primer for PCR

SEQ ID NO:53

Designed oligonucleotide primer for PCR

SEQ ID NO:54

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:57

Designed oligonucleotide primer for PCR

SEQ ID NO:58

Designed oligonucleotide primer for PCR

SEQ ID NO:59

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:60

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:61

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:62

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:63

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:64

Designed oligonucleotide primer for PCR

SEQ ID NO:65

Designed oligonucleotide primer for PCR

SEQ ID NO:66

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:67

Designed oligonucleotide primer for DNA sequencing

SEQ ID NO:70

Designed oligonucleotide primer for PCR

SEQ ID NO:71

Designed oligonucleotide primer for PCR

SEQ ID NO:72

Designed oligonucleotide primer for PCR

SEQ ID NO:73

Designed oligonucleotide primer for PCR

SEQ ID NO:74

Designed oligonucleotide primer for PCR

SEQ ID NO:75

Designed oligonucleotide primer for PCR

SEQ ID NO:76

Designed oligonucleotide primer for PCR

SEQ ID NO:77

Designed oligonucleotide primer for PCR

SEQ ID NO:78

Designed oligonucleotide primer for PCR

SEQ ID NO:79

Designed oligonucleotide primer for PCR

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SEQ ID NO:87

Designed oligonucleotide primer for PCR

SEQ ID NO:88

Designed oligonucleotide primer for PCR